THE ROLE OF BARBITURIC ACID IN THE NUTRITION OF BACILLUS POPILLIAE

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

WILSON HUXLEY COULTER

1968

This is to certify that the

thesis entitled

THE ROLE OF BARBITURIC ACID IN THE
NUTRITION OF BACILLUS POPILLIAE

presented by

WILSON HUXLEY COULTER

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

Major professor

Date November 18, 1968

ABSTRACT

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By

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Growth studies showed that a high concentration of barbituric acid (0.1%) was required for maximum growth of Bacillus popilliae in a synthetic medium, and that this requirement was not replaced by other pyrimidines. However, only a trace amount of that added (~2%) disappeared from the medium during growth. No detectable amount of barbituric acid was degraded into urea and malonic acid, or oxidized by cells harvested from complex media. No effect of barbituric acid was observed on (a) oxidation of glucose or reduced nicotinamide adenine dinucleotide (NADH), (b) production of H_2O_2 by cell extracts during NADH oxidation, or (c) loss in viability of cells. A small amount of ^{14}C from 2-^{14}C-barbituric acid was consistently associated with both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA); and the amount associated with the RNA increased in a linear manner during incubation. A general distribution of the isotope among cell components was not observed. The isotope found in RNA was uniformly distributed throughout the 4s, 16s, and 23s RNA fractions, and control experiments indicated

that at least 10% of this may occur by nonspecific adsorption. None of the ^{14}C from 2-^{14}C-barbituric acid was found associated with mononucleotides following hydrolysis of the RNA. The presence of barbituric acid resulted in very significant stimulation of both nucleic acid and protein synthesis, and it is believed that the stimulatory effect by barbituric acid may be due to a stabilization of the complexes involved in macromolecular synthesis.

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By

Wilson Huxley Coulter

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1968

DEDICATION

This thesis is dedicated to my parents who wisely instilled in me the values of an education.

ACKNOWLEDGMENTS

I would like to express my deepest appreciation to Dr. R. N. Costilow for his generous and patient guidance during the period of this investigation and for his critical evaluation of this dissertation.

I am particularly indebted to Dr. J. A. Boezi of the Department of Biochemistry for his friendly advice and technical guidance with some of the experiments presented in this thesis.

The author also wishes to thank Dr. H. L. Sadoff for his helpful suggestions and discussions during the period of this investigation, and Dr. D. E. Schoenhard for his evaluation of this manuscript. In addition, the author is very grateful to the Northern Utilization Research and Development Division, U.S.D.A., Peoria, Illinois, for financial assistance.

In conclusion, without the patient understanding and willing sacrifices on the part of my wife, Elaine, this investigation would have been impossible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Barbituric acid is required by Bacillus popilliae for growth in a synthetic medium and this requirement is not replaced by the common pyrimidines or purines found in nucleic acids (82). However, it is not required for growth in semisynthetic medium containing "vitamin-free" casein hydrolysate plus a variety of vitamins, or in complex media containing yeast extract (18,82). One would not expect barbituric acid to be present in the latter two media. Thus, the actual role of this compound in the nutrition of this organism is obscure.

There are a number of possibilities for the nutritional response of B. popilliae to barbituric acid viz., (a) utilization by the cell as an energy source, (b) an effect on energy generation either by serving as an alternate electron acceptor or by interfering with the transfer of electrons from flavin to oxygen resulting in reduced H_2O_2 production, (c) alteration of cell permeability, (d) utilization by the cell as a required constituent or precursor of a cell component(s), and (e) regulation of metabolic activity.

It does not appear likely that B. popilliae utilizes this compound as a primary energy source since this organism requires sugar for growth and oxidizes glucose rapidly (63,82). However, some microorganisms can grow in a medium

containing barbituric acid as the main source of carbon and nitrogen (3,86). Also, organisms which were adapted to growth on uracil or thymine could utilize barbituric acid as an oxidizable substrate (3,6,37,50,86). In all of the above cases, barbituric acid was found to be an intermediate in the oxidative catabolism of pyrimidines which these organisms were found to be capable of carrying out. However, it does not appear likely that barbituric acid serves as an energy source in B. popilliae by this pathway since neither barbituric acid nor the other normal pyrimidines were found to serve as energy sources for this organism in media in which barbituric acid was required for growth.

Barbituric acid may have an effect on the energy generation by this organism. Previous findings have shown that H_2O_2 was produced by the soluble electron transport system in cells from late exponential and stationary phase cultures of B. popilliae (64), and that these cells were quite sensitive to the lethal effects of 0.01M H_2O_2 (18). Since no evidence has been found for the presence of a H_2O_2 scavenging system in those cells which produced H_2O_2 (64), the rapid loss in viability observed with this organism may be due to the production of low intracellular concentrations of this compound (18). Hence, one possible role of barbituric acid in synthetic medium was the reduction of H_2O_2 accumulation. It was thought that barbiturate might either serve as an electron acceptor from

reduced flavins, or interfere with their reaction with molecular oxygen.

Since B. popilliae cells loose viability rapidly under some conditions, it appeared possible that barbiturate might stabilize the permeability barrier of the cell; thereby, preventing the leakage of essential metabolites. Amobarbital has been shown to inhibit the uptake of orotic acid into the pool of B. cereus (55). Also, the stimulation of antibiotic production by barbital with some species of Streptomyces may be due to its prevention of early autolysis of the mycelium; resulting in the extension of antibiotic production (25). A similar role by barbituric acid in the growth of B. popilliae appeared worthy of consideration.

Although barbituric acid may be providing the cells of B. popilliae with an essential precursor of a cell constituent(s), this does not appear to be very likely in the case of pyrimidine biosynthesis since normal pyrimidines do not replace the barbituric acid requirement in a synthetic medium (82). Nevertheless, the possibility existed that derivatives of barbituric acid may be formed which may serve as essential intermediates in the synthesis of some macromolecule(s) of the cell.

In view of the previous findings (82) which suggested that barbituric acid was not utilized as a primary energy source or as a precursor to pyrimidine biosynthesis, the most probable role proposed for this pyrimidine in the nutrition of B. popilliae was in the regulation of metabolic

activity. Barbiturates have been found to stimulate the production of antibiotics by certain microorganisms (25,46,56), and to result in the production of elevated levels of alanine racemase in Escherichia coli (31). Also, dihydroorotic dehydrogenase has been found to be inhibited by barbituric acid (27,83), and this may be the reason for the increased levels of unaffected pyrimidine biosynthetic enzymes observed on addition of this compound to cultures of Escherichia coli (5).

The primary aim of this study is to explore the possibilities for the nutritional response of B. popilliae to barbituric acid. Hopefully, this investigation will result in a clarification of the requirement for barbituric acid for growth in synthetic medium.

REVIEW OF LITERATURE

Bacillus popilliae.--*B. popilliae* is a causative agent of "milky disease" of Japanese beetle (*Popillia japonica*) (23). The progress of the disease has been described (4,76). Approximately 2 x 10^9 spores are produced per larvum resulting in a characteristic milky opacity of the infected larvae. At this time death occurs. Since the bacterial spores are highly infective to beetle larvae, a great deal of interest has evolved in developing media which will support the growth and sporulation of the organism *in vitro* to obtain high concentrations of spores for the effective biological control of the host.

Laboratory media have been devised which support the vegetative growth of the organism *in vitro* (18,23,39,77, 82). The highest yield of cells ($\sim$2 X 10^9/ml) has been obtained in a liquid medium containing 1.5% trypticase, 0.5% yeast extract, 0.2% glucose, and 0.6% K_2HPO_4(18). Nevertheless, this organism grows poorly when compared to most other members of the same genus, is characterized by a rapid loss of viability after vegetative growth, and sporulates very poorly if at all on laboratory media (18,67, 68,77,78,79). Low concentrations of spores have been obtained *in vitro* only by the use of specialized techniques which entailed either the collection of cells from solid media into pastes followed by the subsequent transfer of

the pastes to solid sporulation media (78,79), or the addition of activated carbon to a liquid sporulation medium (38). Recently, up to 0.3% sporulation has been attained by spreading dilutions of appropriate strains of vegetative cultures on to acetate agar plates providing there were fewer than 30 colonies per plate (67). Up to 20% sporulation has been observed in the surface colonies of a new varient culture on an agar medium consisting of casein hydrolysate, beef infusion, yeast extract, soluble starch, and trehalose (Sharpe, E.S., and G. St. Julian, Bacterial Proc., p.10, 1967). Due to the lack of success in obtaining adequate yields of spores on laboratory media, a number of studies have been conducted on the nutrition and physiology of this organism.

Dutky (23) first described the organism as facultatively anaerobic but subsequent investigations have shown that oxygen is required for growth (63,68,77). Larval macerates or extracts supported vegetative growth but not sporulation on laboratory media. Optimal growth occurred in the pH range of 7.0 to 7.2 at 32 C. Glucose, mannose, galactose, maltose, and salicin were fermented by the organism (77). The acids produced were adequately neutralized providing a buffer consisting of 0.6% K_2HPO_4 was included in the media (68).

Consistent growth in synthetic medium was observed only if thiamine, biotin, 11 amino acids, 0.1% barbituric acid, 1.0% glucose, and 0.2% K_2HPO_4 were included (82).

Barbituric acid had no effect on growth when included in a vitamin rich semisynthetic medium consisting of 1.5% acid hydrolysate of casein, 1.0% glucose, 0.2% K_2HPO_4, 0.01% DL-tryptophan, and the vitamins p-aminobenzoic acid, biotin, pantothenate, folic acid, myoinositol, niacin, pyridoxine, riboflavin, and thiamine; or when included in medium containing 1.5% yeast extract, 0.2% glucose, and 0.6% K_2HPO_4.

Although liquid media which supported vegetative growth, did not support the sporulation of the organism, spore-like bodies were obtained in a medium containing 4% trypticase, 0.2% K_2HPO_4, 0.1% barbituric acid, 0.02% $MnSO_4 \cdot H_2O$, 0.02% $CaCl_2$, 0.01% DL-tryptophan, and 1 ppm of thiamine·HCL under environmental conditions which resulted in an extension of vegetative cell viability (18). Comparisons of these bodies with vegetative cells and spores produced <u>in vivo</u> indicated that they were more similar to spores since they contained catalase; decreased levels of reduced nicotinamide adenine dinucleotide (NADH) oxidase, pyrophosphatase, and of most of the catabolic and electron transport enzymes; elevated levels of ribosidase; and lower levels of protein, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA). However, they were unlike spores since they lacked heat-resistance, were morphologically and cytologically dissimilar, contained higher concentrations of poly-ß-hydroxybutyric acid and acid-soluble phosphate, and lacked dipicolinic acid (18,59). It is thought that

these spore-like bodies are the end result of abortive sporulation (59).

Physiological studies have indicated that both the Embden-Meyerhof pathway and the hexosemonophosphate pathway are operative in this organism depending on the availability of oxygen. The main products of glucose catabolism are lactic and acetic acids, and one strain of B. popilliae was found capable of oxidizing acetate via the tricarboxylic acid cycle (63). Cell extracts contained a nicotinamide adenine dinucleotide phosphate (NADP) dependent glucose -6-phosphate dehydrogenase, lacked reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and NADPH-nicotinamide adenine dinucleotide (NAD) transfer enzyme, but contained both particulate and soluble NADH oxidase systems. The cytochrome dependent particulate system was prevalent in extracts prepared from younger cells. However, the soluble system was predominant in extracts of older cells and was characterized by H_2O_2 production, flavin dependence, and a lack of sensitivity to azide, cyanide, and carbon monoxide. No evidence was obtained for the presence of a H_2O_2 scavenging system in B. popilliae cells although they produced H_2O_2 (64); and cells were found to be quite sensitive to the lethal effects of 0.01M H_2O_2. The rapid loss in viability observed with this organism may be due to the production of low intracellular concentrations of H_2O_2 (18). The cytochrome system was deficient in cytochrome

c but was found to contain cytochrome b_1 and possibly cytochromes a_1 and a_2 (64).

The physiology of sporogenesis of this organism as well as the role of barbituric acid in the vegetative growth on synthetic medium and in the development of spore-like bodies in sporulation medium remain to be elucidated.

Barbituric acid.--Barbiturates are known for their inhibition of electron transport from NADH to oxygen in the flavoprotein region (12), but their site of inhibition is still controversial (13,24,41,61). Studies conducted on the barbiturate inhibition of D-aspartate oxidoreductase indicated that inhibition was competitive with substrate and that a relatively large substituent group at the C-5 position was necessary for inhibition (11). Data obtained from assays of a large number of enzymes suggested that barbiturates may interact with enzyme-bound flavins. However, in some cases barbiturates may interact with the protein moiety of some enzymes since some non-flavoenzymes were found to be inhibited (29). Although unsubstituted barbituric acid had no effect on the enzymes assayed, it was found to have a maximum effect on the light-induced oxidation of NADH by flavin or riboflavin, and on the photolytic degradation of flavin mononucleotide (FMN) suggesting a direct interaction of barbiturates with flavin (30). Barbituric acid has been found to be an effective inhibitor of two flavoenzymes; viz., NADP-dependent dihydroorotic dehydrogenase

isolated from an aerobic bacterium (83), and the NAD-dependent enzyme isolated from the anaerobe, Zymobacterium oroticum (27). Only in the latter case was NADH oxidase activity associated with the enzyme which was inhibited by barbituric acid in the presence and absence of orotic acid.

Barbiturates have been found to have other effects on a number of microorganisms. Barbital has been shown to result in a stimulation of antibiotic production by some species of Streptomyces. Rifomycin B was produced in higher concentrations without contamination by other complexes in the presence of 0.2% barbital by S. mediterranei (56). Streptomycin production was increased 4.5 fold in the presence of 13.5 x 10^{-3}M barbital (25). The role of barbital is not known but it may result in the stimulation of enzyme systems responsible for antibiotic production or may be responsible for the observed delay in autolysis of the mycelium. Barbital did not act as a precursor of the antibiotic (25,46). Barbital at 0.02M concentration resulted in the production of 2.5 times the normal amount of alanine racemase due to increased pool levels of L- and D-alanine by partial inhibition of D-alanine oxidase in E. coli, without having any effect on the growth of the organism (31). Amobarbital has been shown to inhibit the uptake of orotic acid into nucleic acids of B. cereus by inhibiting its uptake into the pool (55).

The major known role of unsubstituted barbituric acid in microbial metabolism is as an intermediate in the oxidative catabolism of pyrimidines. The organisms in which barbiturates have been found as intermediates in pyrimidine breakdown include Corynebacterium, Mycobacterium (6,36,37, 50), Nocardia (3,50), and a Bacterium species (86). The organisms were isolated by enrichment culture in the presence of the respective pyrimidine. Uracil and thymine were oxidized with the uptake of one atom of oxygen per mole of pyrimidine to barbituric acid and 5-methylbarbituric acid respectively by the same adaptive enzyme (3,36, 37,51). An additional adaptive enzyme was necessary for the deamination of cytosine to uracil (37,87). Barbituric acid and 5-methylbarbituric acid were isolated and identified as the products of purified uracil-thymine oxidase on uracil and thymine respectively (6,37,87). Barbituric acid was degraded anaerobically to urea and malonic acid (3,37, 51), but the corresponding enzyme had no effect on 5-methylbarbituric acid (6,37). Work carried out by Batt and Woods (3) suggested that the enzymes responsible for the degradation of barbituric acid and 5-methylbarbituric acid were induced only by the respective substrate. Biggs and Doumas (6) have clearly shown that methylmalonic acid and urea were the degradation products of 5-methylbarbituric acid.

In many cases considerably less than the theoretical amounts of oxygen uptake and CO_2 evolution were observed

for the complete oxidation of uracil by resting cells (3,37,86). The possibility exists that this may have been due to oxidative assimilation or to the inability of the cells to oxidize malonic acid. However, malonic acid is known to be utilized by some bacteria as a carbon source (37), and bacteria grown on uracil have been shown to be capable of oxidizing malonic acid (86). Nevertheless, the strain of N. carollina studied by Batt and Woods (3) utilized methylmalonic acid but not malonic acid as a carbon source. In addition, the uracil-grown organism was incapable of oxidizing malonic acid and this acid had no effect on the oxidation of uracil, or barbituric acid. The possibility exists that in some cases an active derivative of malonic acid may be formed immediately after the degradation of barbituric acid without the intermediate formation of free malonic acid. Of interest, is the reported interconversion of the CoA derivatives of malonic and acetic acids with the decarboxylation of malonic acid by extracts of Pseudomonas fluorescens (35).

EXPERIMENTAL METHODS

Culture and cultural methods.--Cultures used in this study were obtained from the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Peoria, Illinois. The experiments were conducted using B. popilliae strains NRRL B-2309-P-A (63) and NRRL B-2309M (Sharpe, E.S., and G. St. Julian, Bacterial. Proc., P.10, 1967).

Cultures were maintained on agar slants as outlined by Sylvester and Costilow (82) and in a medium (TYG) consisting of 15 g of trypticase (BBL), 5 g of yeast extract, 2 g of glucose, and 6 g of K_2HPO_4 (pH 7.2 to 7.4) per liter as described by Costilow et al.(18). This medium was dispensed in 250 ml volumes into 500 ml Erlenmeyer flasks. Cultures were incubated at 30 to 32 C on a rotary shaker and transfers made to fresh medium at intervals of 24 to 72 hr.

Some of the experiments were conducted with cultures grown in a synthetic medium designated as S-5 (82). The composition of the medium was (per liter): glucose, 10 g; K_2HPO_4, 2 g; barbituric acid, 1 g; biotin, 2 µg; thiamine. HCL, 400 µg; L-arginine, 400 mg; L-asparagine, 800 mg; L-cystine.HCL, 200 mg; glycine, 200 mg; L-histidine.HCL, 100 mg; L-isoleucine, 50 mg; L-leucine, 100 mg; DL-methionine, 200 mg; DL-phenylalanine, 200 mg; L-proline, 200 mg; DL-serine, 100 mg; DL-tryptophan, 100 mg; L-tyrosine,

100 mg; DL-valine, 200 mg. The initial pH was 7.2 to 7.4. The synthetic medium was prepared in the absence of glucose, biotin, and thiamine, dispensed in 50 ml volumes into 125 ml Erlenmeyer flasks unless otherwise specified and autoclaved at 121 C for 15 min. A solution containing 25 g of glucose, 5 µg of biotin and 1 mg of thiamine per 100 ml was sterilized by filtration through an asbestos Seitz filter pad, and the appropriate amount added aseptically to the cooled medium. A basal synthetic medium (BS) having the same composition of S-5 but prepared without glucose and barbituric acid was used in a number of experiments.

Growth studies.--Growth studies were carried out with both strains of B. popilliae to determine the requirement for barbituric acid in S-5 medium and to see if this requirement could be met by other pyrimidines such as uracil, cytosine, thymine, orotic acid, and diethylbarbituric acid (barbital). The media were prepared as described above with the individual pyrimidines substituted for barbituric acid at a concentration of 0.1%. Inocula were prepared in various ways as described in the Results. Growth was measured by determining the optical density (OD) at 620 mµ with a Beckman model DU spectrophotometer using the growth medium clarified by centrifugation as a blank.

Oxidation and breakdown of pyrimidines by resting cells.--The manometric determinations of O_2 uptake and CO_2 evolution were carried out by the direct method as described by Umbreit, Burris, and Stauffer (85). Unless otherwise noted, all components of the reaction mixture were added to the main compartment of the Warburg flask except substrate which was tipped in from a side arm after thermal equilibrium was attained. In all cases the experiments were run at 30 C and the total volume per flask was 3 ml. The cells were grown in TYG medium, harvested in the cold, washed twice with cold 0.1M phosphate buffer (pH 7.2) and resuspended in the same medium unless otherwise noted.

The production of urea from barbituric acid was estimated manometrically by measuring the amount of CO_2 produced after the addition of urease from a side arm of the Warburg flasks.

Effect of barbituric acid on H_2O_2 production and on reduced nicotinamide adenine dinucleotide (NADH) oxidation.--Strain NRRL B-2309M was grown in TYG medium, washed twice with cold water, and cell extracts prepared with the Nossal cell disintegrator (McDonald Engineering Co., Cleveland, Ohio) using approximately 2 g wet weight of cells and 10 g of glass beads (2 mm average diameter) in a total volume of about 5 ml. The cells were disintegrated for a total of 2 min. After removal of the glass beads by low speed centrifugation, the soluble and particulate fractions

were obtained as described below. Some of the cells were fractionated by the use of a 100 watt Ultrasonic Disintegrator (Measuring and Scientific Equipment Limited, London, England) for a total of 2 min. Over 90% cell breakage was indicated by observation with a phase-contrast microscope.

The soluble and particulate fractions were obtained by centrifugation at 110,000 x g for 3 hr in the Beckman, model L, preparative ultracentrifuge (Spinco Division, Beckman Instruments, Palo Alto, California). The soluble fraction was decanted and the particulate fraction was resuspended in distilled water. The protein content of the fractions was estimated by the method of Lowry et al.(54).

Standard Warburg techniques (85) were used to measure oxygen uptake. The main compartment of the Warburg flasks contained all the components of the reaction mixture with the exception of NADH which was added to one side arm. The other side arm contained 0.2 ml of 4N H_2SO_4 and the gas phase was 100% O_2. The total volume in the flasks was 3 ml. After temperature equilibration, the NADH was tipped in and the reaction run at 30 C for 1 hr after which the acid was tipped in to stop the reaction. The entire contents of the flasks were transferred to test tubes with the aid of 1 ml of water as a wash and the samples titrated for H_2O_2 production by the iodometric method as described by Herbert (40).

Experiments were also conducted in cuvettes in which the oxidation of NADH was measured spectrophotometrically by following the decrease in OD at 340 mμ with a Beckman model DU spectrophotometer.

Effect of barbituric acid on permeability and viability.--Strain NRRL B-2309M was grown in TYG medium for 24 hr and resuspended in various diluents and the amount of RNA leakage determined by the orcinol method (58) as described by Demain, Burg, and Hendlin (20). The cells were harvested and approximately 100 mg wet weight of cells was resuspended in 30 ml of each of the following diluents: distilled water, 0.1M phosphate buffer (pH 7.3), and 0.1M phosphate buffer containing 0.1% barbituric acid (pH 7.3). After 60 min at room temperature, 10 ml aliquots of each suspension were centrifuged at room temperature and the supernatant solutions evaporated to dryness. The residue was dissolved in 1 ml of distilled water and the amount of RNA determined as described above.

The effect of barbituric acid on permeability was also estimated by measuring the pools of glutamic acid and proline. Cells were grown in TYG medium for 20 hr, harvested, washed in BS medium containing 1% glucose and with and without 0.1% barbituric acid respectively, and then added in 1 ml amounts to 125 ml Erlenmeyer flasks containing 14 ml of the same BS medium. U-^{14}C-L-Proline

and U-^{14}C-L-glutamic acid were added to separate flasks of each treatment to give a final activity of 2.04 x 10^5 and 4.66 x 10^6 cpm/μmole respectively. The proline flasks contained S-5 medium which contained 5 mg of L-proline per 100 ml instead of the usual 20 mg. Duplicate 0.1 ml aliquots were removed at various time intervals, one placed into a prechilled tube containing 2 ml of the respective media and the other into a prechilled tube containing 2 ml of 5% trichloroacetic acid (TCA). The TCA precipitate was filtered on to HA type millipore filters (Millipore Filter Corp., Bedford, Mass.) and washed twice with cold 5% TCA. The dried filters were glued to planchets (Planchets, Inc., Chelsea, Mich.) and the cpm determined by using a Nuclear-Chicao thin window gas-flow counter, Model 3037B (Nuclear Instrument and Chemical Corp., Chicago, Ill.) with the counts registered on a Berkeley Decimal Scaler, Model 100 (Berkeley Scientific Corp., Richmond, Calif.) at a high voltage setting of 1250 volts.

The effect of barbituric acid on cell viability was determined by resuspending cells in various diluents followed by the determination of viable counts on solid media by the method of St. Julian et al.(69). Strain NRRL B-2309M was grown in TYG medium for 24 hr, and the cells from 5 ml aliquots harvested aseptically, washed once, and resuspended aseptically in 5 ml of one of the following diluents: TYG medium, S-5 medium, 0.1M phosphate

buffer (pH 7.3), 0.1M phosphate buffer containing 0.1% barbituric acid (pH 7.3), and distilled water. After standing at room temperature for 3 hr, the viable count of each 5 ml aliquot was determined by plating out decimal dilutions of the suspensions in duplicate on TYG agar plates. The colonies on the plates were counted after incubation at 30 C for 4 days.

Incorporation of ^{14}C from 2-^{14}C-barbituric acid into cell components.--An initial experiment was conducted with cells harvested from a 24 hr TYG culture to determine the distribution of the label from 2-^{14}C-barbituric acid in cell fractions. The cells were harvested, washed twice in cold distilled water and resuspended in the same. A 0.5 ml aliquot of the cell suspension was transferred to a 125 ml Erlenmeyer flask containing 10 ml of S-5 medium to which had previously been added sufficient 2-^{14}C-barbituric acid to give a total activity of approximately 2.78×10^5 cpm/μmole. Aliquots of 2 ml were removed at 0, 1.5, and 3 hr; added to prechilled centrifuge tubes and centrifuged. At the same time intervals, 0.5 ml aliquots were removed and placed on previously weighed millipore filters followed by 4 washes with 2 ml of cold S-5 medium. The filters were then dried, their dry weights determined and the radioactivity of the intact cells measured. The centrifuged cells were then extracted by the method of Schneider (73) as follows. The pellets were resuspended in 2 ml of cold 5%

TCA, allowed to stand on ice for 15 min, and centrifuged. The supernatant solutions containing the cold TCA-soluble fractions were each decanted into a test tube with the aid of a glass rod. After an additional wash in cold 5% TCA, the pellets were resuspended in 2 ml of 5% TCA and placed in a boiling water bath for 30 min. The samples were then chilled on ice, centrifuged, and the hot TCA-soluble fractions containing the extracted nucleic acids were decanted into test tubes as described above. The pellets were resuspended in 2 ml of cold distilled water and quantitatively removed with the aid of an additional wash of cold water to previously weighed millipore filters. The filters were then dried, the dry weights of the hot TCA-precipitates determined, and the radioactivities determined.

The ^{14}C content of the wall peptidoglycan fraction of the 3 hr sample was determined by the method of Park and Hancock (62). This procedure was as follows. The hot TCA pellet was washed with 0.01N NH_4HCO_3, resuspended in a small volume of the same, and the volume adjusted to 4 ml after the pH had been adjusted to 8.0. After the addition of crystalline trypsin (400 µg/ml), the sample was incubated at 37 C overnight, washed with 1N NH_3 followed by 3 washes with cold distilled water, and the trypsin-soluble material removed for the determination of radioactivity.

The pellet which contained the wall fraction was then quantitatively removed to a preweighed millipore filter, dried, and the radioactivity determined after the determination of the dry weight.

Experiments were also conducted using the extraction procedure based on that of Schmidt and Thannhauser (72) to determine the effect of chloramphenicol on the incorporation of the isotope into cell components. Aliquots of 1.5 and 2.0 ml volumes were removed from the reaction mixture at 2, 4, and 6 hr, and the samples treated to obtain the cold TCA-precipitates. The precipitates from the 1.5 ml samples were resuspended in cold distilled water and quantitatively transferred with the aid of two 2 ml aliquots of cold water as a wash to previously weighed millipore filters for the determination of ^{14}C uptake from 2-^{14}C-barbituric acid into the total nucleic acid fraction. The cold TCA-precipitates from the 2 ml aliquots were resuspended in 3 ml of 0.3M KOH and incubated at 37 C for 18 hr. The DNA and protein were then precipitated by the addition of equal volumes of cold TCA (10%). The pellets containing DNA and protein were resuspended in cold 5% TCA and quantitatively transferred with the aid of two washes with 2 ml of cold 5% TCA to preweighed millipore filters for the determination of ^{14}C uptake from 2-^{14}C-barbituric acid into the DNA plus protein fractions. The TCA was removed from the supernatant solutions containing the cold TCA-soluble fractions by extraction with ether before the determination

of the ^{14}C content. An attempt was made to separate the RNA mononucleotides on a Dowex-1-formate column to be described later. Radioactivity determinations were made in the gas-flow counter.

An attempt was made to determine the fate of the isotope quantitatively by use of liquid scintillation counting. The cells were harvested from 24 hr TYG cultures, washed twice in cold S-5 medium, and incubated for 6 hr in S-5 medium containing 2-^{14}C-barbituric acid before the removal of samples for fractionation. A determination of the ^{14}C content of the medium was made before and after the incubation period in an attempt to measure the depletion of the isotope from the medium. After 6 hr the cells were washed 3 times with cold S-5 medium and resuspended in a total volume of the cold medium equal to that of the reaction mixture. To determine the amount of radioactivity in intact cells, a 1 ml aliquot was removed to a tube containing 0.5 ml of hydroxide of Hyamine 10 X (Packard Instrument Company, Inc., Downers Grove, Illinois), and aliquots removed for counting after incubation at 37 C for approximately 3 hr. To determine the distribution of radioactivity in the cold TCA-soluble, ethanol-ether soluble, and TCA-insoluble fractions, a 1 ml aliquot was removed from the washed suspension and added to a tube containing 2 ml of cold 5% TCA. After standing on ice for 15 min, the precipitate was centrifuged, washed once with 2 ml of cold 5% TCA, and the supernatants combined for the determination

of radioactivity of the cold TCA-soluble fraction. The cold TCA-insoluble precipitate was extracted with ethanol-ether (1:1) for 15 min at 40 C, washed once with an equal volume of the same, and the supernatants combined for the determination of the radioactivity of the ethanol-ether soluble fraction. The precipitate was then dissolved in 0.5 ml of hydroxide of Hyamine as described above and the radioactivity of the acid insoluble precipitate determined. The remainder of the original washed suspension (30 ml) was extracted with cold TCA and the insoluble precipitate fractionated to obtain RNA mononucleotides and the DNA plus protein fraction by the method of Littlefield and Dunn (52) as follows. The acid insoluble pellet was re-suspended in 0.3M KOH, incubated at 37 C for approximately 24 hr, neutralized to pH 7.0 with perchloric acid and the volume determined after the removal of the potassium perchlorate precipitate by centrifugation. Aliquots were removed at this time to determine the radioactivity of the protein plus nucleic acid fraction. The solution was then brought to pH 4.0 with acetic acid and absolute ethanol added to a final concentration of 67% (w/v). The precipitated DNA and protein was removed by centrifugation and the radioactivity of the RNA solution determined. RNA mononucleotides were separated by paper electrophoresis as described later. In this particular experiment, the radioactivities of the fractions were determined by adding aliquots of the liquid fractions to polyethylene vials

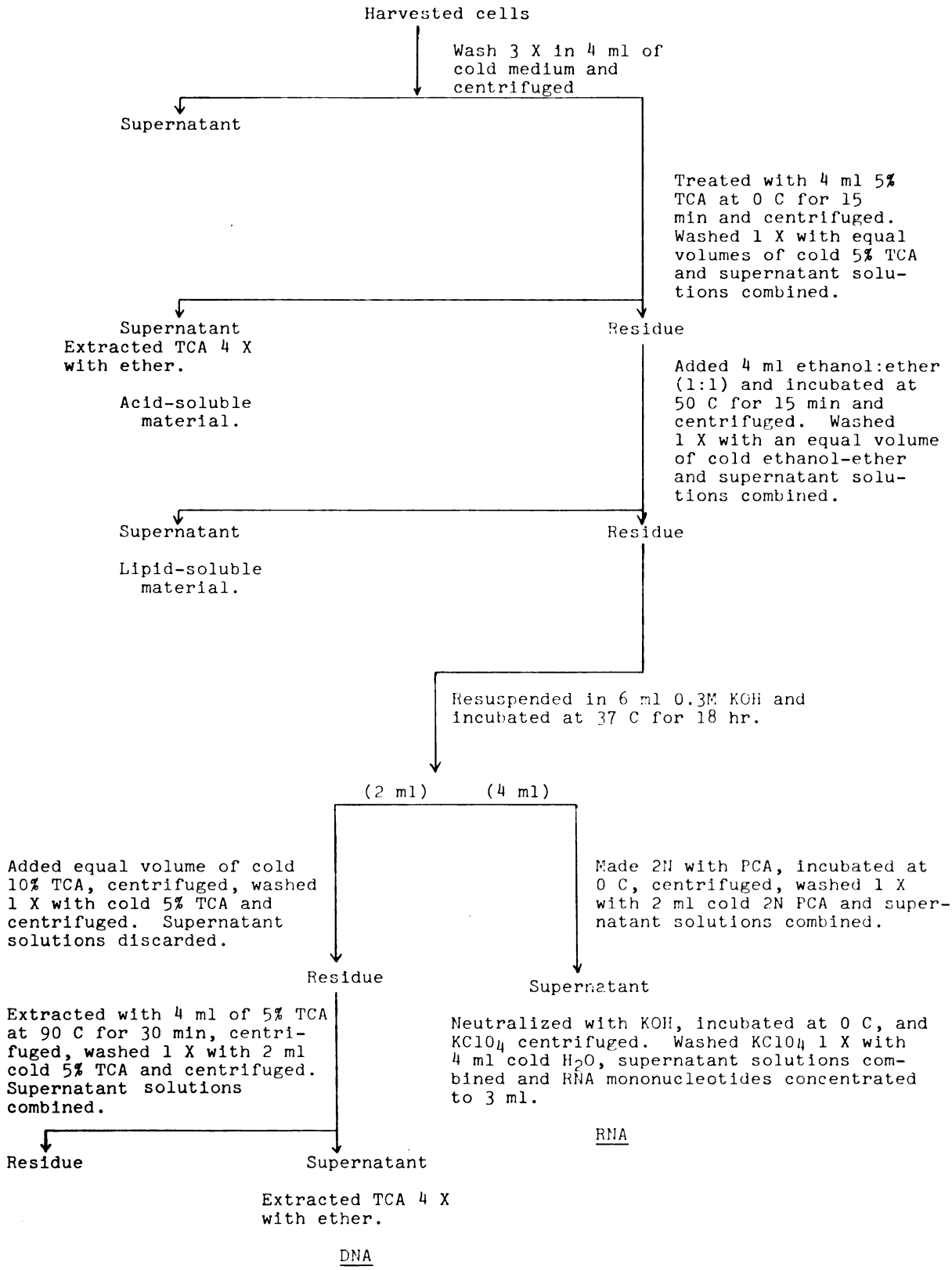
(Packard Instruments Company, Inc., Downers Grove, Illinois) followed by the determinations of the radioactivities in a Tri-Carb Scintillation Spectrometer, model 314-DC (Packard Instruments Company, Inc., LaGrange, Illinois). Then a standard amount of ^{14}C-glutamic acid was added to each vial, the vials recounted, and the determinations corrected for efficiency, quenching, and background.

Experiments were also carried out to determine the amount of label present per mg of RNA and DNA. In these cases the amounts of RNA and DNA present were estimated colorimetrically by the orcinol (58) and diphenylamine (9) reactions respectively.

In some cases, the amounts of RNA and DNA synthesized were determined by measuring the amount of radioactive phosphorus (^{32}P) uptake into these fractions. The samples were treated by the method of Schmidt and Thannhauser (72) including an ethanol-ether (1:1) extraction as previously described. Perchloric acid (PCA) was used instead of TCA to acidify the alkaline hydrolysate after alkaline digestion. The procedure used is outlined in Scheme 1.

To determine the amount of label from 2-^{14}C-barbituric acid in highly purified RNA from cells grown in S-5 medium containing the radioactive pyrimidine, the RNA was extracted from the labeled cells according to the method described by Asano (2). The cells were extracted twice with 1% sodium dodecyl sulphate (SDS) in 0.01N sodium acetate

SCHEME 1


Harvested cells
Wash 3 X in 4 ml of cold medium and centrifuged
Supernatant
Treated with 4 ml 5% TCA at 0 C for 15 min and centrifuged. Washed 1 X with equal volumes of cold 5% TCA and supernatant solutions combined.
Supernatant
Extracted TCA 4 X with ether.
Acid-soluble material.
Residue
Added 4 ml ethanol:ether (1:1) and incubated at 50 C for 15 min and centrifuged. Washed 1 X with an equal volume of cold ethanol-ether and supernatant solutions combined.
Supernatant
Lipid-soluble material.
Residue
Resuspended in 6 ml 0.3M KOH and incubated at 37 C for 18 hr.
(2 ml)
(4 ml)
Added equal volume of cold 10% TCA, centrifuged, washed 1 X with cold 5% TCA and centrifuged. Supernatant solutions discarded.
Made 2N with PCA, incubated at 0 C, centrifuged, washed 1 X with 2 ml cold 2N PCA and supernatant solutions combined.
Residue
Supernatant
Extracted with 4 ml of 5% TCA at 90 C for 30 min, centrifuged, washed 1 X with 2 ml cold 5% TCA and centrifuged. Supernatant solutions combined.
Neutralized with KOH, incubated at 0 C, and $KClO_4$ centrifuged. Washed $KClO_4$ 1 X with 4 ml cold H_2O, supernatant solutions combined and RNA mononucleotides concentrated to 3 ml.
RNA
Residue
Supernatant
Extracted TCA 4 X with ether.
DNA

(ph 5.0). In each case an equal volume of water-saturated phenol was added and the aqueous layer containing the RNA removed after stirring for 10 min in the cold. The aqueous fractions were then re-extracted twice with phenol, made to 0.3M with sodium acetate (pH 5.0) and the RNA precipitated by addition of an equal volume of cold absolute ethanol. The precipitation was repeated one time. After resuspending the RNA in a small volume of 0.01M sodium acetate and making the solution 0.002M with respect to magnesium chloride, the contaminating DNA was removed by treating with 10 μg/ml of deoxyribonuclease (Worthington Biochemicals, Freehold, New Jersey, electrophoretically purified) at 25 C for 10 min. After making to 0.3M with sodium acetate, the RNA was obtained by two ethanol precipitations as described above. The RNA was then redissolved in a small volume of 0.01M sodium acetate containing 0.05M sodium chloride (pH 5.2) and the supernatant solution obtained after centrifugation at 10,000 rev/min for 10 min. After determination of the amount and the spectrum of the RNA present as well as the radioactivity of the RNA, a suitable dilution of the RNA was fractionated on a sucrose gradient (5 to 20% w/v) prepared as described by Britten and Roberts (8). Centrifugation was carried out in a SW-39 rotor at 39,000 rev/min for 5.5 hr at 3 C in a Beckman, model L-2 ultracentrifuge. The RNA fractions were then collected and the OD at 260 mμ determined. To determine the radioactivity

of the RNA, the RNA was precipitated by the addition of 5 ml of cold 10% TCA after the addition of 2 drops of salmon sperm DNA as a carrier, and chilled on ice for at least 30 min. The preciptates were collected on B-6 membrane filters (Carl Schleicher and Schuell Co., Keene, New Hampshire) and washed 2 X with 5 ml of cold 5% TCA. The filters were then air-dried, placed in glass scintillation vials and the radioactivity determined in a Packard Tri-Carb Liquid Spectrometer, Model 3003, after the addition of 5 ml of scintillation fluid consisting of 15.1 g/gallon of BBOT [2,5-bis-2-(5-tert-butylbenzoxozolyl)-thiophene] (Nuclear-Chicago, Des Plaines, Ill.) in toluene.

For determination of the amount of label present in highly purified DNA from cells grown in S-5 medium containing 2-^{14}C-barbituric acid, the DNA was extracted from the labeled cells by the method of Saito and Muira (71). The cells were extracted with a buffer solution containing 0.05M Tris-HCL, 0.5M EDTA, and 0.5% SDS (pH 8.0) in a round bottom flask which was rotated at room temperature for 15 min. An equal volume of water-saturated phenol was added, and the sample centrifuged at 10,000 rev/min after extraction for 15 min. After the removal of the aqueous layer containing the DNA with a large-bore Pasteur pipette, one-half volume of SDS-buffer solution was added to the phenol fraction and the sample extracted for 10 min. Following centrifugation, an equal volume of phenol was added to the combined aqueous fractions and the samples

re-extracted. The sample was centrifuged, made to 0.3M with sodium acetate (pH 8.0), the DNA wound on to a glass rod from the interface after the careful addition of a 2 volumes of absolute ethanol, and the DNA redissolved in a small volume of a solution of 0.1M Tris-HCL and mM EDTA (pH 8.0). The contaminating RNA was removed by treatment with 20 µg/ml of pancreatic ribonuclease at 30 C for 30 min. The DNA was then rewound on to a glass rod after the addition of a 2 X volume of absolute ethanol, redissolved in a small volume of a buffer solution containing 0.05M Tris-HCL and 0.5mM EDTA (pH 8.0), and made to 0.3M with sodium acetate. The DNA was rewound on to a glass rod after the addition of a 0.54 volume of isopropanol, redissolved in a small volume of buffer, and the quantity, spectrum, and radioactivity of the DNA determined as described in the previous paragraph. The remainder of the ethanolic solution from the first ethanol precipitation step was treated as described previously for the extraction of RNA, and the radioactivity of this fraction determined.

Where indicated, attempts were made to separate the RNA mononucleotides by anion exchange through a Dowex-1-formate column as described by Cohn (15), and Cohn and Volkin (16), and by paper electrophoresis on Whatman 3MM paper at 32 volts/cm for 1 hr using 0.05M ammonium formate (pH 3.5) as described by Smith (75), and Markham and Smith (57).

Incorporation of isotope from 2-^{14}C-barbituric acid into protein was checked by the method of Schneider (73). Samples were removed into test tubes containing 2 ml of 5% TCA, heated at 90 C for 30 min, and the precipitated protein collected on millipore filters with the aid of two washes of 2 ml of cold 0.01N HCL. The radioactivity on the dried filters was determined in the gas-flow counter.

In some experiments, the incorporation of isotope from 2-^{14}C-barbituric acid into nucleic acid was studied by the use of the extraction procedure described by Hanawalt (32). Aliquots were centrifuged, resuspended in 2 ml of cold 5% TCA on ice for 15 min to remove the cold TCA-soluble fraction, and then resuspended in 4 ml of ethanol-ether (1:1) for 12 min at 50 C to remove the lipid-soluble material. The sample was then resuspended in 2 ml of cold 5% TCA, collected on B-6 type filters and washed with two 2 ml volumes of cold 0.01N HCL. After the determination of radioactivity in the DNA plus RNA fraction, the filters were placed in polyethylene scintillation vials containing 3 ml of 2N KOH and incubated at 37 C for 18 hr. The samples were then acidified by the addition of an equal volume of cold 50% TCA (it was found that the addition of 10% TCA as suggested in the reference was insufficient to acidify the samples). The precipitate was then collected on millipore filters for the determination of radioactivity in the DNA as described above. The

The radioactivity of the RNA was then determined by difference.

Effect of barbituric acid on RNA and DNA synthesis.-- Cells of strain NRRL B-2309M were harvested from TYG cultures, washed once in cold BS medium with 1% glucose and with and without 0.1% barbituric acid respectively, and resuspended in a small volume of the same medium. Aliquots of these suspensions were then inoculated into flasks containing the same BS medium, and ^{32}P as $K_2H^{32}PO_4$. At various time intervals, RNA and DNA synthesis was determined in filtrates of samples which were removed and extracted by the procedure described by Hanawalt (32) as outlined in the previous paragraph. The radioactivity of the filtrates was determined in a gas-flow counter.

Effect of barbituric acid on protein synthesis.-- Cells of strain NRRL B-2309M were harvested from TYG cultures as described in the previous paragraph, and inoculated into flasks containing BS medium with 1% glucose and with and without 0.1% barbituric acid respectively, and a ^{14}C-labelled amino acid. Unless otherwise indicated, aliquots were removed into test tubes containing 2 ml of cold 5% TCA, heated at 90 C for 30 min, chilled on ice, and the precipitates collected quantitatively on millipore filters with the aid of two 2 ml volumes of 0.01N HCL as a wash. The dried filters were then glued to planchets and the radioactivity of the precipitates determined in a gas-flow counter.

RESULTS

Growth studies.--The results from experiment 1 (Table 1) indicate that barbituric acid was required by both strains of B. popilliae tested for growth in S-5 medium and that the requirement for barbituric acid was not completely met by any of the other pyrimidines tested. Thymine appeared to partially replace the barbiturate requirement by strain NRRL B-2309-P-A, but growth in the presence of 0.1% thymine with strain NRRL B-2309M was less than in the control lacking pyrimidine. With both strains, the growth obtained with the other pyrimidines was equal to or less than that observed in the control flask. The growth observed with strain NRRL B-2309M in the control medium may have been due to the small amount of barbituric acid added to the test flasks during inoculation, the long incubation time, better adaptation to growth in S-5 medium because of previous growth in this medium, or due to characteristics of the particular strain. Using a shorter incubation time (Experiment 2, Table 1), very little growth of this strain was noted in the absence of barbiturate. Also, it is evident from this experiment that a relatively high level of barbiturate is necessary for maximum growth response.

TABLE 1.--Effect of various pyrimidines on the growth of two strains of B. popilliae in a synthetic medium[a].

Pyrimidine added	OD_{620}	
	NRRL B-2309-P-A	NRRL B-2309M
Experiment No. 1:		
None	0.021	0.193
Barbituric acid	0.092	0.467
Uracil	0.012	0.150
Cytosine	0.013	0.183
Thymine	0.048	0.088
Barbital	0.022	--
Orotic acid	--	0.172
Experiment No. 2:		
None	--	0.029
0.1% barbituric acid	--	0.320
0.05% barbituric acid	--	0.161
0.01% barbituric acid	--	0.068

[a]In Experiment No. 1, the NRRL B-2309-P-A inoculum was prepared by aseptically centrifuging 10 ml aliquots from 24 hr TYG cultures followed by resuspension in 10 ml of sterile saline.(0.85%). Two ml of this suspension was added to flasks of BS medium containing 1% glucose and the pyrimidine to be tested at a concentration of 0.1% and the OD at 620 mμ determined after 72 hr incubation. Flasks containing the same BS media were inoculated with 2% inocula of strain NRRL B-2309M from 24 hr S-5 cultures and the OD determined after 84 hr incubation. Experiment No. 2 was conducted by observing the growth of a 34 hr culture of strain NRRL B-2309M after 3 consecutive transfers of 10% inocula every 24 hr in the S-5 medium containing the various levels of barbituric acid.

The results of experiments to determine the level of glucose required for maximum growth in the presence of 0.1% barbituric acid (Table 2) indicate that a maximum growth response was not observed with glucose levels lower than 1% in the synthetic medium. These results substantiate those presented by Sylvester and Costilow (82).

The growth pattern of NRRL B-2309M in S-5 and TYG media (Fig. 1) indicate that the growth rate of this organism is about 3 times greater in TYG medium (generation time $\approx$ 2.2 hr) than in S-5 medium (generation time $\approx$ 6.0 hr). In both media, the cessation of the exponential rate of growth is evident at 12 to 15 hr. Previous work carried out in this laboratory suggested that the exponential rate of growth continued for about 24 hr. However, at that time the cultures were transferred every 48 to 72 hr, whereas for the present experiment the cultures had been transferred every 24 hr for several days in the respective media before inoculation into the test flasks. Microscopic examination of the cultures during logarithmic growth indicated that the cells in the S-5 medium consisted of many long and pleomorphic strands whereas the cells in TYG cultures were typical short rods occurring singly and in pairs as shown by Mitruka et al.(59).

Oxidation and breakdown of pyrimidines by resting cells.--Strain NRRL B-2309-P-A was incapable of oxidizing barbituric acid as substrate (Table 3) whether the cells

TABLE 2.--Effect of glucose level on the growth of *B. popilliae* NRRL B-2309-P-A in a synthetic medium[a].

Glucose added	OD_{620}
1.0%	0.092
0.5%	0.070
0.1%	0.034
0.05%	0.031

[a]BS medium containing 0.1% barbituric acid and various levels of glucose were inoculated with 2 ml aliquots of strain NRRL B-2309-P-A as described under Table 1. The OD of the cultures was determined after 72 hr incubation.

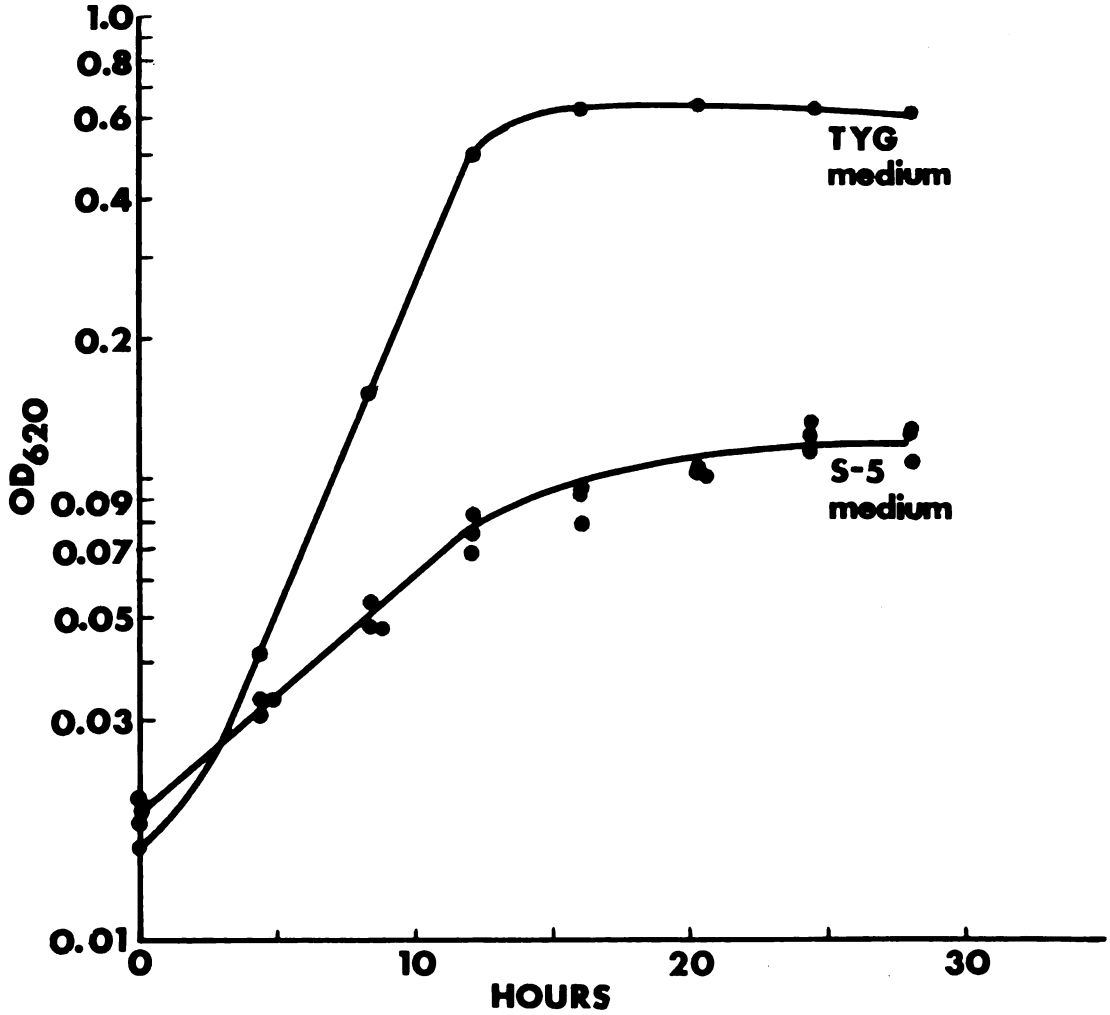


Figure 1.--Growth pattern of strain NRRL B-2309M in TYG and S-5 media. The media were prepared and dispensed in 125 ml Erlenmeyer flasks at 50 ml per flask. The TYG and S-5 media were inoculated with 5 and 10 ml aliquots from respective 24 hr cultures in the same media. The flasks were incubated on a rotary shaker at 30 C, and 1 TYG culture and 3 S-5 cultures removed at the designated time intervals for the determination of growth as described in the Experimental Methods.

TABLE 3.--Ability of strain NRRL B-2309-P-A to oxidize barbituric acid and uracil[a].

Substrates	Age of cells (days)	Experiment No. 1	Experiment No. 2		
		Q_{O_2}	Q_{O_2}	Q_{CO_2}	RQ
Endogenous	1	2.88	5.56	8.04	1.45
Glucose		13.81	18.09	19.08	1.05
Barbituric acid		2.47	4.82	6.84	1.42
Uracil		--	5.38	7.54	1.40
Endogenous	3	--	4.51	5.35	1.19
Glucose		--	11.00	11.91	1.08
Barbituric acid		--	4.07	4.55	1.12
Uracil		--	4.61	5.35	1.16
Endogenous	5	--	0.73	1.64	2.25
Glucose		--	2.28	2.68	1.18
Barbituric acid		--	0.36	0.87	2.42
Uracil		--	0.82	1.32	1.61

[a]The reaction mixtures contained 200 µmoles of phosphate buffer (pH 7.2) in the main compartment of the Warburg flasks, 0.2 ml of 20% KOH in the center well, 0.2 ml of 4N H_2SO_4 in one side arm, and the following substrates in the other side arm as indicated above: glucose, 33.5 µmoles; barbituric acid, 50.0 µmoles; and uracil, 4.5 µmoles. The experiments were conducted as described in the Experimental Methods and the acid tipped in from the side arm at the end of the incubation period for the determination of evolved CO_2. Experiment No. 1 was conducted with cells of strain NRRL B-2309-P-A harvested from TYG cultures whereas Experiment No. 2 was done with the same strain grown in sporulation medium as described by Costilow et al. (18). The reaction mixtures contained 21.9 mg (dry weight) of cells in Experiment No. 1 and 16.4 mg of cells in every case in Experiment No. 2.

were grown in TYG medium or in a sporulation medium where barbituric acid is required for the development of spore-like bodies (18). In every case the Q_{O_2} and Q_{CO_2} values observed with barbituric acid or uracil as substrate were approximately equal to or less than the endogenous values. Barbituric acid did not have a significant effect on the ability of cells of this same strain to oxidize glucose as substrate (Table 4). These observations were made both with cells previously grown in TYG medium and in sporulation medium.

Barbituric acid did not have any effect on glucose oxidation by cells of strain NRRL B-2309M grown in TYG medium and resuspended in BS medium (Table 5) nor were these cells able to oxidize barbituric acid in the absence of glucose. The latter observation was made whether the cells were resuspended in BS medium or phosphate buffer. It is also apparent that the cells were not able to oxidize any other component in the BS medium since the Q_{O_2} observed were less than those observed in the presence of phosphate buffer. Similarly, cells from 24 and 48 hr cultures of strain NRRL B-2309M grown in S-5 medium were not able to oxidize barbituric acid in the absence of glucose and the presence of barbiturate did not have a significant effect on the ability of these cells to oxidize glucose (Experiment 2, Table 5). The differences observed may be attributed to errors introduced into the computation of these results since only very small amounts

TABLE 4.--Effect of barbituric acid on the ability of resting cells of strain NRRL B-2309-P-A to oxidize glucose[a].

Substrates	Culture	Q_{O_2}	Q_{CO_2}	RQ
Endogenous	A	2.06	--	--
Glucose + barbituric acid		24.50	25.00	1.02
Glucose - barbituric acid		20.10	--	--
Endogenous	B	1.96	--	--
Glucose + barbituric acid		12.91	13.71	1.06
Glucose - barbituric acid		14.59	15.90	1.09
Endogenous	C	0.45	1.48	--
Glucose + barbituric acid		4.54	4.79	1.06
Glucose - barbituric acid		5.09	6.12	1.20

[a]Strain NRRL B-2309-P-A was grown as follows: culture A, harvested after 24 hr growth in TYG medium; culture B, cells were grown for 24 hr in TYG medium, transferred to B-4 medium after which a 20% inoculum was transferred to sporulation medium after 48 hr and the cells incubated for 24 hr before harvesting; culture C, same as for culture B except that the sporulation medium was inoculated with 10% inoculum and incubated for 48 hr before harvesting. The Warburg cups were prepared as described under Table 3 with the exception that the amount of barbituric acid added where indicated was 7.8 µmoles. The dry weights of cells added were: culture A, 22.4 mg; culture B, 14.8 mg; and culture C, 8.1 mg.

TABLE 5.--Effect of barbituric acid on the oxidative ability of *B. popilliae* NRRL B-2309M.[a]

Substrates	A			B		
	Q_{O_2}	Q_{CO_2}	RQ	Q_{O_2}	Q_{CO_2}	RQ
Experiment No. 1:						
Endogenous						
Control	--	--	--	1.8	2.2	--
Plus barbituric acid	--	--	--	1.5	1.8	--
BS medium (with glucose)						
Control	12.5	14.4	1.15	--	--	--
Plus barbituric acid	13.6	15.4	1.13	--	--	--
BS medium (no glucose)						
Control	1.2	1.9	1.58	0.9	1.7	--
Plus barbituric acid	2.2	3.3	1.50	1.1	2.1	--
Experiment No. 2:						
Endogenous						
Control	0	0	--	2.1	0	--
BS medium (with glucose)						
Control	44.1 (41.9)	29.7 (44.3)	0.67 (0.94)	19.5	18.7	0.96
Plus barbituric acid	49.4 (42.8)	56.7 (46.3)	1.15 (1.08)	24.8	36.2	1.46
BS medium (no glucose)						
Control	1.1 (4.8)	0 (4.7)	-- (--)	2.4	1.6	--
Plus barbituric acid	0.9 (3.8)	0 (8.7)	-- (--)	2.4	1.3	--

[a]The Warburg cups contained 2.3 ml of BS medium or 0.1M phosphate buffer (pH 7.3) in the main compartment and 0.5 ml of cell suspension was tipped in from a side arm after temperature equilibration. The glucose and barbituric acid were present where indicated at the normal levels for S-5 medium. In Experiment No. 1, strain NRRL B-2309M was grown in TYG medium for 24 hr before harvesting. The Warburg cups contained (A) 26.4 mg, and (B) 50.6 mg dry weight of cells respectively. In Experiment No. 2, the Warburg cups contained (A) 2.9 mg dry weight of cells harvested from 24 hr S-5 cultures, and (B) 8.0 mg dry weight of cells harvested from 48 hr S-5 cultures. Enclosed in brackets are values obtained from a duplicate experiment with cells harvested from 24 hr S-5 cultures where 2.8 mg dry weight of cells were present per Warburg cup.

of cells (2.9 and 2.8 mg dry weight of cells at 24 hr and 8.0 mg dry weight of cells at 48 hr) were obtainable from S-5 media for these experiments. The Q_{O_2} and Q_{CO_2} values observed with cells grown in S-5 medium were approximately 3 times greater than those observed with cells grown in TYG medium. This may be a reflection of the requirement of a higher amount of glucose in the synthetic medium.

Urea was not produced from barbituric acid by cells of strain NRRL B-2309-P-A harvested from TYG medium (Table 6). The amount of CO_2 evolved in the presence of barbituric acid and urease was less than the endogenous CO_2 evolution. Hence, it seems likely that these cells do not contain an enzyme such as barbiturase capable of degrading barbituric acid to urea and malonic acid.

Effect of barbituric acid on H_2O_2 production and on NADH oxidation.--Barbituric acid did not have a significant effect on the production of H_2O_2 by the soluble extract prepared from cells from 24 hr cultures either in the presence or absence of flavin adenine dinucleotide (FAD) (Experiment 1, Table 7). The addition of NaN_3 resulted in a significant increase in the percent of observed oxygen uptake accountable for in the H_2O_2 produced, thus indicating a slight contamination in the soluble extract of a particular electron transport system. The data indicate that the addition of FAD greatly stimulated oxygen uptake and H_2O_2 production by this system. The data obtained with cells

TABLE 6.--Production of urea from barbituric acid.[a]

Substrate	µl CO_2 evolved	Percent of Theoretical
Endogenous	98.7	--
Barbituric acid, 9.4 µmoles	84.7	--
Barbituric acid, 9.4 µmoles	85.4	--
Urea standard, 15 µmoles	339.4	101.00
Urea standard, 10 µmoles	219.7	98.1
Urea standard, 10 µmoles (urease boiled 10 min.)	15.8	7.1
Urea standard, 5 µmoles	130.2	116.3
Urea standard, 2 µmoles	33.0	73.7
Urea standard, 1 µmoles	23.1	103.1

[a]Strain NRRL B-2309-P-A was grown in TYG medium for 24 hr, washed in 0.1M phosphate buffer (pH 7.3), and added along with substrate and 200 µmoles of the buffer to the main compartment of the Warburg flasks. The side arms contained 0.2 ml of 4N H_2SO_4 and a solution containing 4 mg of urease respectively. The flasks were incubated at 30 C for 75 min after temperature equilibration, followed by the addition of urease and an additional 10 min incubation period. Acid was tipped in to liberate the CO_2 produced and the final reading taken after a further 10 min incubation period. The standards were run as described above but in the absence of cells and with the indicated levels of urea.

TABLE 7.--Effect of barbituric acid on H_2O_2 production.[a]

Extract	Cofactor added	µmoles O_2 uptake	µmoles H_2O_2	% of observed O_2 in H_2O_2
Experiment No. 1:				
Soluble	+BA	4.81	2.01	41.8
Soluble	-BA	4.73	2.29	48.1
Soluble	+BA, NaN_3	3.57	3.68	103.1
Soluble	-BA, NaN_3	3.61	3.56	98.6
Soluble	+BA, FAD	11.07	9.23	83.4
Soluble	-BA, FAD	10.80	9.95	92.1
Soluble	+BA, NaN_3, FAD	10.99	11.44	104.1
Soluble	-BA, NaN_3, FAD	11.16	11.56	103.6
Experiment No. 2:				
Soluble	+BA	2.75	1.43	51.9
Soluble	-BA	2.78	2.48	89.4
Particulate	+BA	12.42	0.94	7.6
Particulate	-BA	12.60	1.00	7.9

[a]Experiment No. 1 was conducted with extracts prepared from 24 hr TYG cultures by ultrasonic disintegration. The main compartment of the Warburg flasks contained 5 mg (protein) of soluble extract, 190 µmoles of phosphate buffer (pH 7.3) and where indicated 12.5 µmoles of barbituric acid (BA), 30 µmoles of NaN_3, and 0.6 µmoles of FAD. One side arm contained 24 µmoles of NADH as substrate and the other side arm contained 0.2 ml of 4N H_2SO_4. Experiment No. 2 was conducted with extracts prepared from 35 hr TYG cultures in the Nossal disintegrator and the particulate fraction of a cell extract (3.4 mg protein) and 14.8 µmoles of barbituric acid were included where indicated. The amount of H_2O_2 produced was determined as described in the Experimental Methods.

Endogenous samples were run in the absence of NADH. No oxygen uptake or H_2O_2 production was observed.

from 35 hr cultures (Experiment 2, Table 7) indicate that lower amounts of H_2O_2 were produced in the presence of barbiturate, but the differences observed are probably of doubtful significance due to the low values obtained.

Similar results were obtained when NADH oxidation was measured spectrophotometrically, with the cell extracts utilized above and with extracts of cells harvested from 48 hr cultures. Again, barbituric acid did not have a significant effect on NADH oxidation in the presence and absence of NaN_3 by the soluble extracts tested. (Table 8). The addition of FAD to the soluble fraction of cells obtained from 35 hr cultures greatly stimulated the NADH oxidase activity. This extract was prepared by use of the Nossal Disintegrator and this may account for the small differences observed in the activities of the various extracts in the absence of FAD. The data obtained with the particulate fraction from cells harvested from 35 hr cultures indicate that FAD and barbiturate had slightly inhibitory effects on the activity of this fraction and that this system was completely sensitive to NaN_3.

The data presented in this section substantiate those presented by Pepper and Costilow (64) and do not give any consistent indication that barbituric acid may be involved with the soluble or particulate electron transport systems. The pyrimidine certainly was not oxidized to an extent necessary for it to serve as an energy source, nor did it appear to serve as an alternate electron acceptor.

TABLE 8.--Effect of barbituric acid on NADH oxidation.[a]

Extract	Cofactor added	Activities*		
		A	B	C
Soluble	+BA	0.091	0.024	0.052
Soluble	-BA	0.092	0.033	0.054
Soluble	+BA, NaN_3	0.066	0.017	0.029
Soluble	-BA, NaN_3	0.068	0.024	0.034
Soluble	+BA, FAD		0.447	
Soluble	-BA, FAD		0.520	
Soluble	+BA, NaN_3, FAD		0.507	
Soluble	-BA, NaN_3, FAD		0.467	
Particulate	+BA		0.367	
Particulate	-BA		0.431	
Particulate	-BA, FAD		0.349	
Particulate	+BA, NaN_3		0	
Particulate	-BA, NaN_3		0	

[a]Experiments were conducted with extracts prepared from (A) 24 hr cultures, (B) 35 hr cultures, and (C) 48 hr cultures grown in TYG medium. With extracts from 24 hr cells, the cuvettes contained 0.21 µmoles of NADH as substrate, 1.09 mg (protein) of soluble extract, 300 µmoles of phosphate buffer (pH 7.3), and 15.6 µmoles of barbituric acid (BA) and 30 µmoles of NaN_3 where indicated in a total volume of 3 ml. In experiments with extracts from 35 hr cells, 1.0 mg (protein) of soluble extract was included in the absence of FAD whereas 0.05 mg (protein) of soluble extract was included in the presence of FAD which was present at a concentration of 0.6 µmoles. With the particulate extract from these cells, the experiments were conducted as described above with the exception that 60 µmoles of NaN_3 was included where indicated. The amount of particulate extract (protein) included was 0.17 mg. Experiments with the soluble extracts from 48 hr cells were conducted as described for extracts from 24 hr cells with the exception that the cuvettes contained 1.30 mg (protein) of soluble extract. The extracts from 24 and 48 hr cells were prepared by ultrasonic disintegration whereas the extracts from 35 hr cells were prepared in the Nossal disintegrator.

*OD at 340 mµ per min per mg (protein) of extract.

Effect of barbituric acid on permeability and viability.--Barbituric acid may have some stabilizing effect on the permeability barrier of B. popilliae since approximately 1/3 of the RNA was observed in the supernatant from cells suspended in phosphate buffer alone (Table 9). However, the significance of this difference is questionable; since, (a) the method involved concentrating a relatively large volume of supernatant to 1 ml followed by the determination of RNA, and (b), the viable count of samples resuspended in phosphate buffer alone was the same as in phosphate buffer plus barbituric acid. In contrast, when cells were suspended in water both RNA leakage and loss in viability were dramatically increased.

Attempts at measuring the amino acid pool labelled with radioactive glutamic acid or proline were unsuccessful, (Fig. 2), since the counts obtained from samples washed with the S-5 medium were not significantly higher than those samples washed in cold 5% TCA. It should be noted that these cells were previously grown in TYG medium. Hence, the possibility exists that resuspension of these cells in S-5 medium may have been sufficient to release the amino acid pool. Nevertheless, the results indicate that a marked difference occurred in the uptake of labelled amino acid between samples suspended in the presence and absence of barbituric acid irrespective of whether the label was in proline which is required for growth or in glutamic acid which is not required for growth in S-5

TABLE 9.--Effect of various diluents on RNA excretion and cell viability.[a]

Diluent	µg RNA/10 ml supernatant	Viable count/ml
TYG medium	--	1.4×10^8
S-5 medium	--	5.4×10^7
0.1M phosphate (pH 7.3)	94	2.6×10^8
0.1M phosphate containing 0.1% barbituric acid (pH 7.3)	32	3.4×10^8
Water	238	1.0×10^3

[a]Strain NRRL B-2309M was grown in TYG medium for 24 hr, harvested, and resuspended in the indicated diluent. After 1 hr standing at room temperature, the amount of extracellular RNA was determined as described in the Experimental Methods. The viable counts were determined as described in the Experimental Methods after standing at room temperature for 3 hr.

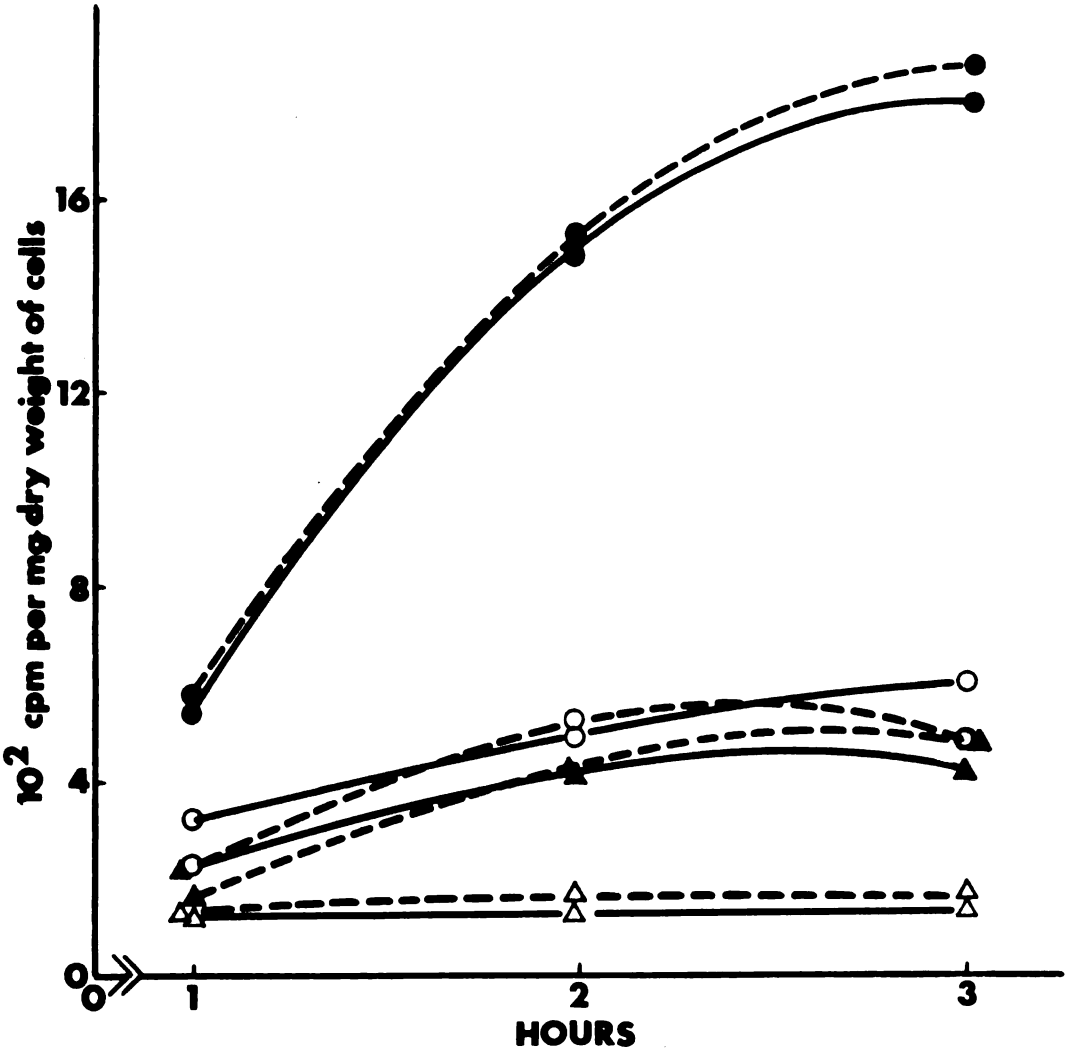


Figure 2.--Effect of barbituric acid on the glutamic acid and proline pools of strain NRRL B-2309M harvested from 20 hr TYG cultures. The cells were washed in BS media with 1% glucose and with and without 0.1% barbituric acid respectively, and resuspended in 15 ml of the same medium. U-^{14}C-L-Glutamic acid and ^{14}C-L-proline were present in the media at approximate specific activities of 4.66×10^6 and 2.05×10^5 cpm/μmole respectively. Duplicate 0.1 ml aliquots were removed at the designated times and treated as described in the Experimental Methods. (●,o) ^{14}C-glutamic acid with and without barbituric acid respectively; (▲,Δ) ^{14}C-proline with and without barbituric acid respectively; (——) cells washed with S-5 medium; (---) cells washed with cold 5% TCA.

medium. The large difference in total radioactivity incorporated between the two amino acids reflects the 20 X difference in specific activity of the acids. These results suggest that barbituric acid may have an effect on the uptake of precursors into cellular macromolecules.

Incorporation of ^{14}C from 2-^{14}C-barbituric acid into cell components.--Preliminary experiments in which the uptake of barbituric acid from the medium was observed by determining the loss in absorbancy at 257 mμ indicated that very little barbiturate was taken up or broken down by the cells since no difference in the absorbancies could be detected after growth in S-5 medium. Hence, barbituric acid uptake was determined by using radioactive barbituric acid.

An initial experiment with 2-^{14}C-barbituric acid (Fig. 3) indicated that the label from barbiturate was rapidly taken up into the cold TCA-soluble pool during the first 1.5 hr of incubation in S-5 medium, but the pool level did not change appreciable thereafter. However, a linear uptake of the label from barbiturate into the nucleic acid fraction (hot TCA-soluble fraction) occurred during the entire incubation period of 3 hr. At 3 hr the percentage of the total radioactivity of the cell fractions found in the hot TCA-precipitate was less than 5.2% and this is not believed to be significant. The high, initial counts of the intact cells may have resulted from

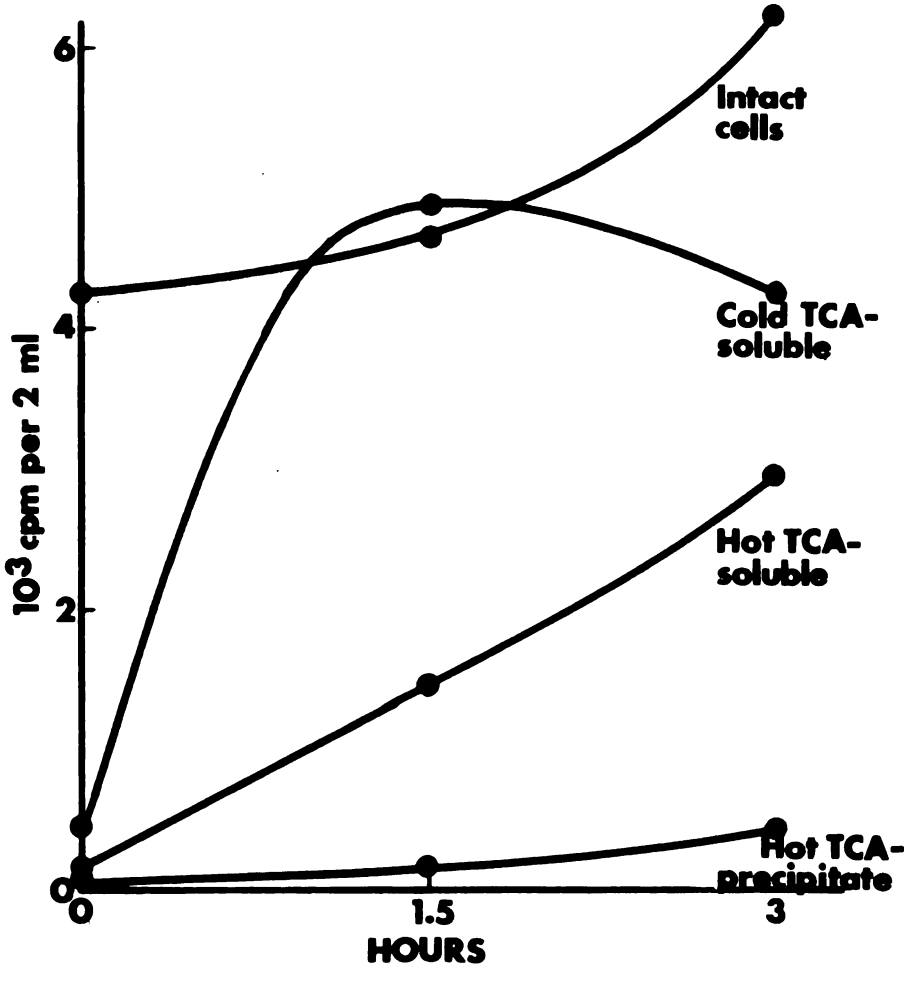


Figure 3.--Initial experiment on the uptake of label from 2-^{14}C-barbituric acid into cells of strain NRRL B-2309M harvested from 24 hr TYG cultures. The cells were washed with water and added to previously prepared S-5 medium containing approximately 2.78 x 10^5 cpm/µmole of 2-^{14}C-barbituric acid. Two ml aliquots were removed and fractionated by the Schneider procedure (73) as described in the Experimental Methods. The hot TCA-precipitate of the 3 hr sample was treated by the method of Park and Hancock (62) as described in the Experimental Methods to obtain the trypsin-soluble and trypsin-insoluble fractions. Over 94% of the total radioactivity of the two fractions was present in the trypsin-soluble fraction. The value for the hot TCA-precipitate at 3 hr is the total of the radioactivities of the two fractions. A 2 ml aliquot of the cell suspension contained 10.9 mg dry weight of cells.

adsorption of the radioactive barbituric acid on the cells during the filtration operation, if the intitial uptake was quite rapid.

Further evidence that the incorporation of isotope from 2-^{14}C-barbituric acid into macromolecules was limited primarily to the nucleic acids was obtained by use of chloramphenicol (Fig. 4). The high level (500 µg/ml of chloramphenicol used had no significant effect on the uptake of ^{14}C from 2-^{14}C-barbituric acid into the cold TCA-soluble fraction of cells, nor into the fraction containing DNA plus protein. However, it did inhibit incorporation into the cold TCA-precipitate, which should have included all the nucleic acids plus protein. The inhibitory effect became greater with time. Such an effect could be accounted for by the inhibition of RNA synthesis after extended incubation (48). Attempts at determining the location of the label in the RNA mononucleotides by separation on a Dowex-1-formate column were unsuccessful although this technique was very successful in the separation of standard solutions of mononucleotides.

Results from experiments carried out to give an estimate of the amount of label from 2-^{14}C-barbituric acid incorporated into the RNA and DNA of resting cells indicated that approximately 8.4×10^{-2} and 1.2×10^{-2} µatoms of ^{14}C from barbiturate were taken up per mg of DNA and RNA respectively. These estimates suggest that more of the label was present in the DNA than in the RNA. However, the

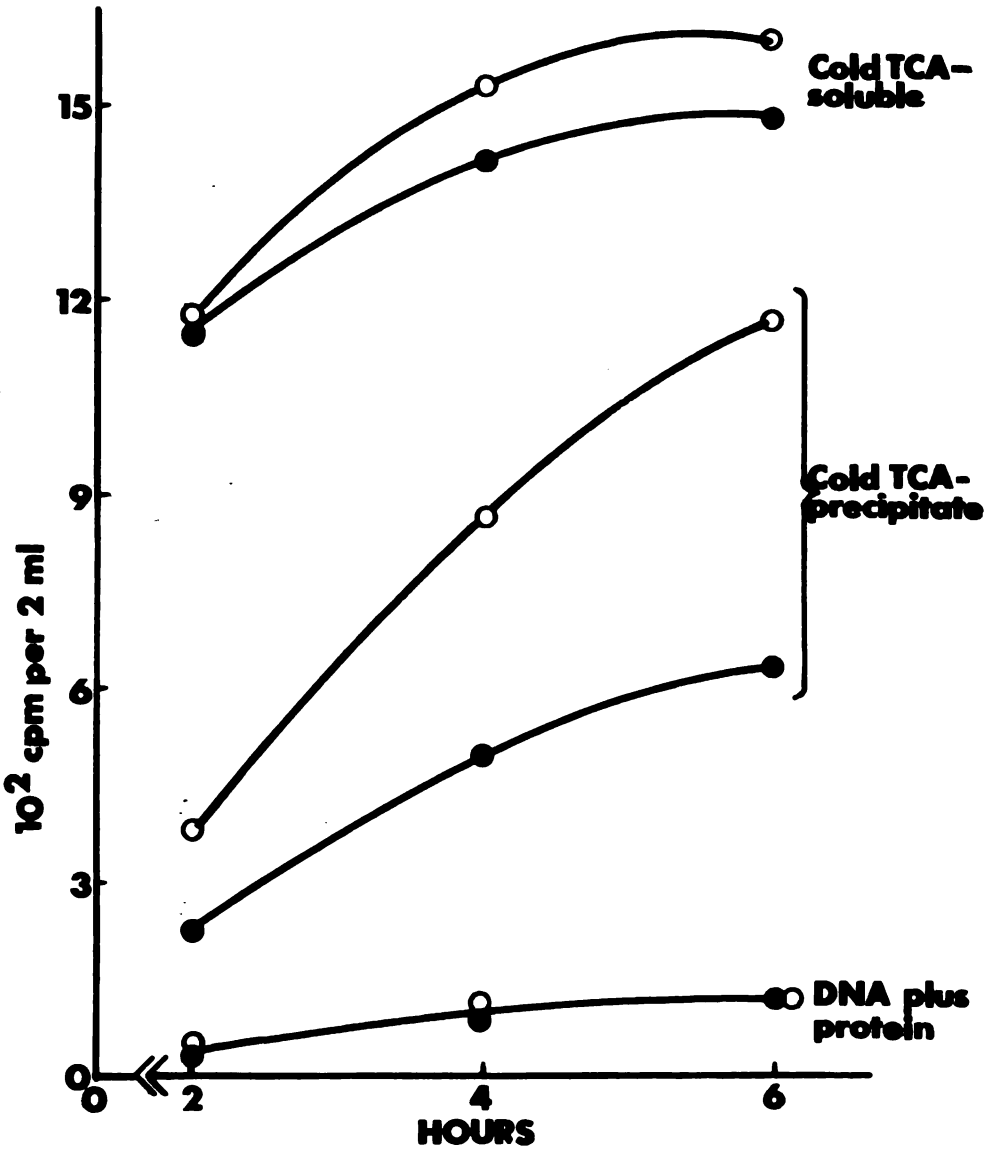


Figure 4.--The effect of chloramphenicol on the uptake of ^{14}C from 2-^{14}C-barbituric acid by cells harvested from 24 hr TYG cultures, washed in cold S-5 medium and resuspended in S-5 medium containing approximately 5.55 x 10^5 cpm/μmole of 2-^{14}C-barbituric acid. Two ml aliquots were removed and fractionated by the Schmidt and Thannhauser procedure (72) as described in the Experimental Methods. A 2 ml aliquot contained 6.2 mg dry weight of cells at the beginning of the experiment. (●) with chloramphenicol at a final concentration of 500 μg/ml; (o) without chloramphenicol.

possibility exists that the DNA fraction was contaminated with low amounts of radioactive RNA or small amounts of 2-^{14}C-barbituric acid carried over in the extraction procedure. Since the amount of DNA present (as assayed colorimetrically) was about one-tenth of the RNA determined, a small contamination of this fraction would result in an over-estimation of the μatoms of ^{14}C present per mg of DNA.

A more complete fractionation of cells was then performed in order to obtain a more quantitative estimate of the distribution of the ^{14}C from 2-^{14}C-barbituric acid in cells. Radioactive measurements were made on these fractions by liquid scintillation counting with appropriate corrections for quenching. About 1% of the label was taken up by the cells over 6 hr (Table 10). The radioactivity present in the intact cells accounted for 81.4% of the observed decrease in the medium and 68.3% of the radioactivity of these cells was recovered in the cell fractions based on the totals of the acid soluble, ethanol-ether soluble, and the pH 7.0 soluble fractions. The latter fraction represents the constituents in the acid insoluble fraction after alkaline hydrolysis and subsequent adjustment of the pH to 7.0. The radioactivity of the RNA fraction amounted to 89.6% of that of the pH 7.0 soluble fraction which provided further evidence that very little of the isotope was associated with the DNA and protein.

TABLE 10.--Distribution of ^{14}C from 2-^{14}C-barbituric acid among the cell fractions.[a]

Sample	dpm/ml of cell suspension
S-5 medium, 0 hr	2,849,000
S-5 medium, 6 hr	2,820,000
Cell washes	70,110
Intact cells	22,960
Cold TCA-soluble fraction and wash	9,650
Ethanol-ether soluble fraction and wash	950
Cold TCA-insoluble fraction	3,780
pH 7.0 soluble fraction	5,092
RNA	4,562
CMP + AMP area	1,806
GMP + UMP area	698
"X" area	440

[a]Cells were grown and harvested from TYG cultures as described under Fig. 3. The cells were fractionated by the Schmidt and Thannhauser procedure (72) including an ethanol-ether extraction step, and the RNA obtained by the method of Littlefield and Dunn (52) as described in the Experimental Methods. The 2-^{14}C-barbituric acid was present in the reaction mixture at a total activity of 3.65×10^5 dpm/µmole and the concentration of cells used was 6.26 mg dry weight per ml. The RNA obtained was concentrated to 3.5 ml, and a total of 0.66 ml electrophoresed with appropriate standards in 20 µl amounts as described in the Experimental Methods. Areas corresponding to those presented in the Table were eluted with water, concentrated to 0.5 ml, and aliquots counted. All counts were corrected to dpm/ml of the original cell suspension.

The wet weight of the cells used in this experiment was estimated to be 31.3 mg/ml since the dry weight of cells present in the reaction misture was 6.26 mg/ml. Assuming their specific gravity to be 1.1 g per ml, the volume of this amount of cells would be approximately 2.9 µl. Hence, the dpm per ml of cell volume would be 5.4×10^6 ($1.57 \times 10^4 / 2.9 \times 10^{-3}$). This estimate was made using the sum of the dpm from the acid soluble, ethanol-ether soluble, and the pH 7.0 soluble fractions; since, it was possible that the method used in solubilizing the intact cells and the acid insoluble fraction for liquid scintillation counting may have resulted in an underestimation of the radioactivity in these fractions due to incomplete solubilization. Also, the amount of radioactivity determined in the intact cells may be inaccurate as an estimate of the intracellular concentration of label due to possible adsorption to the cell surface and incomplete washing of the cells on the filter. Since the amount of radioactivity present in the medium at 0 time was 3.65×10^5 dpm per µmole of 2-^{14}C-barbituric acid, the estimated µmoles of barbiturate per ml of cell volume was 14.8 ($5.4 \times 10^6 / 3.65 \times 10^5$), assuming that the pyrimidine was taken up without degradation of the molecule. The concentration of barbituric acid initially present in the medium was approximately 8 µmoles per ml. This suggests that the cells may be able to concentrate the pyrimidine

intracellularly to about 2 X that present in the extracellular medium.

Although uracil is the most closely related to barbituric acid of the pyrimidines in RNA, the results with the RNA mononucleotides (Table 10) indicated that uridylic acid (UMP) was not the major product formed from barbituric acid. In fact, the ^{14}C levels in the area corresponding to cytidylic acid (CMP) plus adenylic acid (AMP) were almost 3 X that in the area corresponding to guanylic acid (GMP) plus UMP. Of particular interest, was the component designated in the Table as "X" which had an electrophoretic mobility in the buffer system of about twice that of UMP. The "X" component did not absorb ultraviolet (u/v) light on the paper. However, the amount of isotope present in the corresponding area from the 20 µl sample which was separated by electrophoresis was only 75 dpm. This was equivalent to the radioactivity in about 2.1×10^{-4} µmoles of barbiturate. According to Block, Durrum, and Zweig (7), one would need about 4×10^{-2} µmoles of pyrimidine per cm^2 spot for detection under u/v light. Hence, we would have had to separate the RNA mononucleotides in about 4 ml of solution to obtain a sufficient amount of this component in one spot to see u/v absorption. This would correspond to about 15,000 dpm of material. Thus, this component would be very difficult to obtain in sufficient quantity and purity for identification by the present methods.

An analysis of the acid soluble fraction from cells incubated with radioactive barbituric acid showed that 56% of the activity of this fraction corresponded to barbituric acid (Table 11). However, 22.8% of the ^{14}C was associated with a u/v absorbing component corresponding to UMP in electrophoretic mobility. Also, 10.6% of the label was associated with the "X" component referred to earlier. The high percentage of recovery indicated that these components represented essentially all of the isotope present in the pool.

Since the associated radioactivity with UMP in the acid soluble fraction was not observed in the UMP derived from RNA after alkaline hydrolysis, an experiment was conducted to determine if there was an alteration of the radioactive barbituric acid in the uninoculated medium. These results are shown in Table 12. It is apparent that there was an accumulation of a component in the S-5 medium which had the same electrophoretic mobility as UMP during the duration of the experiment. Since a similar accumulation was not noted in the phosphate buffer containing barbituric acid, this component may represent some sort of complex formed between the pyrimidine and some of the amino acids present in the synthetic medium. In any case, these results indicate that the observed radioactivity associated with UMP in the acid soluble fraction may have been due to the formation of a component with similar electrophoretic mobility in the medium, since in some cases

TABLE 11.--Uptake of ^{14}C from 2-^{14}C-barbituric acid into the TCA-soluble fraction of cells harvested from TYG cultures.[a]

Sample		cpm/20 µl
Cold TCA-soluble fraction		544
(After electrophoresis):	Barbituric acid area	305
	UMP area	124
	"X" area	58
Percent recovery		89.5

[a]Cells were harvested from 24 hr TYG cultures and resuspended in S-5 medium as described under Fig. 3. After 6 hr incubation the cold TCA-soluble fraction was extracted and 20 µl electrophoresed as described in the Experimental Methods.

TABLE 12.--Alteration of barbituric acid in uninoculated phosphate buffer and S-5 medium.[a]

Incubation time (days)	Percent at origin area	Percent at barbituric acid area	Percent at UMP area
Phosphate buffer:			
0 (unautoclaved)	1.2	98.2	0.6
0 (autoclaved)	8.3	89.8	1.9
1	7.6	86.3	6.1
2.5	7.5	89.2	3.3
5.5	7.9	85.4	6.7
S-5 medium:			
0 (unautoclaved)	1.1	98.1	0.8
0 (autoclaved)	8.3	88.1	3.6
1	8.2	78.5	13.3
2.5	7.5	74.1	18.4
5.5	7.9	57.3	34.8

[a]Flasks containing 0.1% barbituric acid in 0.1M phosphate buffer (pH 7.3) and complete S-5 medium were prepared containing approximately 2.52×10^5 cpm/µmole of 2-^{14}C-barbituric acid and sterilized. Aliquots were removed aseptically and 20 µl electrophoresed in 0.05M ammonium formate (pH 3.5) along with standard solutions of barbituric acid and UMP. Strips were then cut from these samples and the radioactivity corresponding to the areas occupied by barbituric acid and UMP as well as that present at the origin determined. The percentages in the Table are the percentages of the total count found in each of the three areas.

the synthetic medium was not used on the same day that it was made up. The association of a small amount of radioactivity at the origin may help to explain the presence of radioactivity at the origin of electrophoresis conducted with RNA mononucleotides if this component was capable of associating with the RNA in some manner. The formation of these components in the medium is not believed to have any relation to the ultimate effect of barbituric acid on B. popilliae since no variations were observed in the growth of this organism in fresh media as compared to growth in media after several days storage.

Results from parallel experiments on the effect of actinomycin D on RNA synthesis and on the incorporation of label from 2-^{14}C-barbituric acid into the RNA fraction are shown in Table 13. The antibiotic inhibited RNA synthesis as measured by ^{32}P incorporation by 90.2% whereas the uptake of the ^{14}C label into this fraction was inhibited by only 52.3%. The reason for this difference may be that a relatively lower amount of the ^{14}C was taken up into the RNA.

The previous experiments had been conducted with cells which had been freshly transferred from a complex medium to a synthetic medium. This could interrupt the synthesis of a number of macromolecules. Therefore, a series of experiments were conducted with cells grown in both a synthetic and complex medium. Parallel studies of nucleic acid synthesis were conducted using ^{32}P as

TABLE 13.--Effect of actinomycin D on the uptake of ^{14}C from 2-^{14}C-barbituric acid into RNA.[a]

Sample	Time (hr)	cpm, ^{32}P	cpm, ^{14}C
Control	0	106	52
Actinomycin D		75	42
Control	3	10,291	532
Actinomycin D		1,006	253

[a]Cells were grown for 6 hr in TYG medium and resuspended in 15 ml of S-5 medium with the phosphate decreased to 20 mg per liter, and the buffer increased by adding Tris.HCL, 0.01M (pH 7.3). Duplicate flasks were prepared which contained 2-^{14}C-barbituric acid and ^{32}P at final concentrations of 2.33×10^5 and 1.01×10^7 cpm/µmole respectively. Flasks were also included which contained 25 µg of freshly prepared actinomycin D per flask. Aliquots of 0.3 and 0.5 ml were removed at 0 and 3 hr from the flasks containing the ^{32}P and ^{14}C isotopes respectively and processed by Hanawalts method (32) as described in the Experimental Methods to obtain the RNA + DNA fraction for the determination of radioactivity.

$K_2H^{32}PO_4$ and 2-^{14}C-barbituric acid. In all experiments in which cells were grown in the presence of radioactive barbituric acid, the medium was used within 24 hr of preparation.

Very little of the isotope from barbituric acid was taken up by cells grown in either S-5 or TYG medium as evidenced by the depletion of approximately 2 and 0% respectively of the radioactivity during the growth of the organism. The cells grown in TYG medium took up only about one-third of the ^{14}C taken up by cells grown in S-5 medium (Table 14). This might be expected since barbiturate is not required for growth in TYG medium. There were also differences in the distribution of the label among the fractions. Thus, with TYG grown cells, the percentage of the ^{14}C in the cold TCA-soluble and lipid fractions was higher and the percentage in the nucleic acids lower than with cells grown in S-5 medium. Approximately the same relative amount of ^{32}P was taken up by the cells whether grown in S-5 or TYG medium with or without barbituric acid. This is also reflected in the similarities of the percentage of the total cpm found in the various fractions of cells from the two media.

Since the amount of RNA and DNA present was assayed by the orcinol and diphenylamine reactions, the µatoms of ^{14}C from 2-^{14}C-barbituric acid and the µatoms of ^{32}P incorporated into RNA and DNA per mg of the respective nucleic acid could be determined as shown in Table 15. The µatoms

TABLE 14.--Uptake of ^{32}P and ^{14}C from 2-^{14}C-barbituric acid into growing cells.[a]

Media	Total cpm of cell fractions/ mg cell RNA	Percent of total cpm in			
		Cold TCA-soluble	Lipids	RNA	DNA + protein
With 2-^{14}C-barbituric acid:					
S-5	6,524	16.6	2.2	52.3	28.9
TYG + barbituric acid	2,149	32.3	12.9	39.8	15.0
With ^{32}P:					
S-5	263,746	9.2	5.0	73.3	10.4
TYG + barbituric acid	232,215	9.3	5.0	76.7	9.1
TYG - barbituric acid	244,474	10.2	5.8	75.9	8.1

[a]Strain NRRL B-2309M was grown in TYG medium (the phosphate level was reduced to 0.2% and the medium contained 0.1% barbituric acid where indicated) and S-5 medium with the respective isotope for 24 hr and the cells fractionated as shown in Scheme 1 in the Experimental Methods. The radioactivities were determined after drying the samples on planchets in the gas-flow counter and the amounts of RNA and DNA present assayed colorimetrically as described in the Experimental Methods. The cpm/μmole of 2-^{14}C-barbituric acid was 1.1×10^5, and for ^{32}P was 0.7×10^5 - 0.9×10^5.

TABLE 15.--Calculated μatoms of ^{14}C and ^{32}P per mg of RNA and DNA.[a]

	RNA			DNA		
	(A)	(B)	(C)	(A)	(B)	(C)
With 2-^{14}C-barbituric acid:						
S-5	3.2×10^{-2}	-	1.4	5.2×10^{-2}	-	1.0
TYG + barbituric acid	8.1×10^{-3}	-	0.3	2.7×10^{-2}	-	1.0
With ^{32}P:						
S-5	-	2.3	-	-	5.1	-
TYG + barbituric acid	-	2.5	-	-	2.6	-
TYG - barbituric acid	-	2.6	-	-	2.3	-

[a]The calculations were made from data obtained from the experiment described under Table 14.

(A) μatoms of ^{14}C per mg.
(B) μatoms of ^{32}P per mg.
(C) (A)/(B) x 100: This calculation represents the μatoms of ^{14}C from 2-^{14}C-barbituric acid taken up into the nucleic acid per 100 μatoms of ^{32}P incorporated.

of label from barbituric acid taken up per 100 μmoles of RNA and DNA mononucleotide formed was then calculated by assuming that the ^{32}P data were a true measure of the amounts of RNA and DNA formed. This assumption was particularly accurate in the case of RNA. By taking the average molecular weight of a mononucleotide to be 400, one would expect 2.5 μmoles of mononucleotide per mg of RNA, a value which corresponds very closely to that observed from the ^{32}P data. The reason for the 2 X higher value obtained for DNA from cells grown in S-5 medium is not known. The cells grown in the synthetic medium contained approximately one-fourth as much DNA as RNA. Since the diphenylamine reagent has a fairly high specificity for deoxypentose (21), a small amount of contamination of the DNA fraction with RNA would result in an overestimation of the amount of ^{32}P taken up per mg DNA. It is apparent that cells grown in TYG medium took up only one-fourth as much ^{14}C from 2-^{14}C-barbituric acid per 100 μmoles of RNA mononucleotide as cells grown in S-5 medium.

Table 16 shows the results of an attempt to separate the RNA mononucleotides electrophoretically. From the ^{32}P data it is apparent that an amount of "X" similar to that of the other mononucleotides was synthesized. However, if this component corresponded to a barbiturate mononucleotide or if barbiturate was a required precursor for its formation in synthetic medium, one would expect the corresponding value obtained from the ^{14}C data to be much higher since

TABLE 16.--Microatoms of ^{32}P and ^{14}C estimated in various mononucleotides.[a]

Media	cpm/20µl sample found in areas corresponding to:					
	CMP	AMP	GMP	UMP	"X"	Percent recovery
With 2-^{14}C-barbituric acid:						
S-5	21	7	9	23	27	91.2
TYG + barbituric acid	27	17	14	26	13	71.2
With ^{32}P:						
S-5[b]	750	633	966	717	606	84.5
TYG + barbituric acid[b]	3495	3954	5778	3660	3381	80.9
TYG - barbituric acid	1041	938	1646	1008	1291	82.5

[a]The mononucleotides were isolated from the RNA of cells treated as described under Table 14 by the methods described in the Experimental Methods.

[b]Since the ^{32}P-labelled mononucleotides were concentrated to 3.0 ml and the ^{14}C-labelled mononucleotides were concentrated to 1.0 ml, the actual values obtained with the former were multiplied by 3 so that the values would be comparable.

the unknown component would contain one μatom of ^{14}C from barbituric acid per μmole of mononucleotide. Since this was not observed, the fate of the label from barbituric acid in RNA remains unknown.

To further confirm that the isotope from 2-^{14}C-barbituric acid was truly incorporated into the nucleic acid rather than just associated with the cell fraction in which it was found and to determine the component(s) of RNA with which it was associated, highly purified RNA was prepared from cells grown in fresh S-5 medium with radioactive barbituric acid and fractionated on a sucrose gradient. The spectrum of the purified RNA was very similar to that reported by Swift (81), and the 260/280 ratio was found to be 1.82. The results (Fig. 5) revealed that the ^{14}C from 2-^{14}C-barbituric acid was taken up uniformly into the 4s, 16s, and 23s RNA. This is obvious from the fact that the radioactivity peaks corresponded so closely with the absorbency peaks. This would be possible only if the specific activity of each fraction was essentially the same. In addition, these results give the most conclusive evidence so far that a small but significant amount of the label from 2-^{14}C-barbituric acid is either incorporated or very tightly bound to the RNA.

The amount of barbituric acid equivalent to the ^{14}C incorporated per mg of RNA was estimated from the average cpm (427) per OD_{260}. Since one OD_{260} unit corresponds to about 50 μg of RNA, the activity of the RNA was approximately

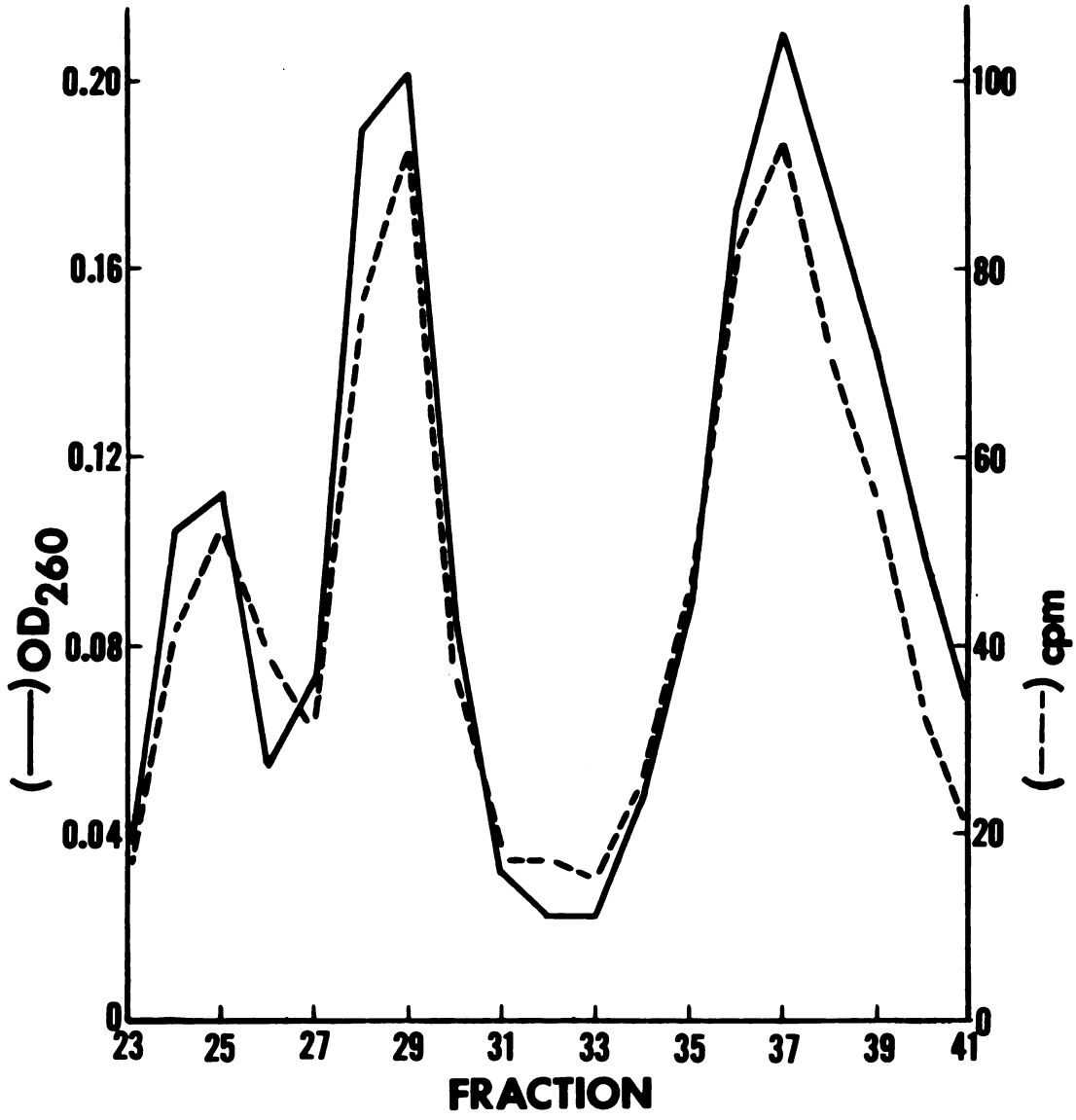


Figure 5.--Incorporation of ^{14}C from 2-^{14}C-barbituric acid into RNA fractions separated by centrifugation through a sucrose gradient. Cells of strain NRRL B-2309M were grown in 200 ml of S-5 medium containing 2-^{14}C-barbituric acid at a final approximate activity of 4.16×10^5 cpm/μmole. The cells were harvested after 24 hr growth and the RNA extracted and fractionated on a sucrose gradient as described in the Experimental Methods. The radioactivities of the fractions were determined by liquid scintillation counting. The OD at 620 mμ of the cell suspension after 24 hr was 0.153.

8420 cpm per mg of RNA. The specific activity of the radioactive barbituric acid was approximately 3.0×10^5 cpm/μmole, hence, the μatoms of ^{14}C from barbiturate taken up per mg of RNA was about 2.8×10^{-2}. This value is very close to that obtained in the previous experiment.

Highly purified DNA was also prepared from cells grown in fresh S-5 medium containing radioactive barbituric acid and analyzed for radioactivity. The 260/280 ratios of the RNA and DNA isolated from these cells were 1.87 and 1.68 respectively. In this experiment the μatoms of ^{14}C from 2-^{14}C-barbituric acid taken up per mg of RNA and DNA into these macromolecules were 2.6×10^{-2} and 1.5×10^{-2} respectively. These results suggest that less radioactivity was associated with the DNA than with the RNA fraction. However, the small amount of DNA analyzed could result in considerable error.

Control experiments were conducted with the RNA isolated by phenol extraction and these results are shown in Table 17. It is apparent that the label in the isolated RNA was completely released from polymer by pancreatic ribonuclease and alkaline hydrolysis. Similar experiments could not be conducted with the DNA since only 13.2 μg was obtained and the complete sample was precipitated with TCA for the determination of ^{14}C content.

Since the amount of ^{14}C from the labelled barbiturate found in the RNA and DNA of cells grown in synthetic medium in which barbiturate was required for growth was extremely

TABLE 17.--Effect of pancreatic ribonuclease and alkaline hydrolysis on RNA isolated by phenol extraction.[a]

RNA	cpm	Percent of control
Experiment No. 1:		
Control	396	--
Enzyme-treated	29	7.3
KOH-treated	31	7.8
Experiment No. 2:		
Control	198	--
KOH-treated	2	1.0

[a]RNA isolated by phenol extraction was incubated in 25 µl aliquots with 10 µg/ml of pancreatic ribonuclease at 30 C for 30 min and with 0.3M KOH at 37 C for 13 hr. The samples were then precipitated and collected on membrane filters as described in the Experimental Methods, and the radioactivities determined. Experiment No. 1 was conducted with the RNA isolated for sucrose gradient centrifugation, and Experiment No. 2 was conducted with the RNA isolated from cells from which DNA was isolated.

small and did not appear to be associated with a specific RNA or mononucleotide, the function of the barbiturate could not be that of a required precursor. In fact, the possibility still remained that none of the label was incorporated. Such small amounts could be tightly bound to the nucleic acids. Accordingly, experiments were conducted with RNA and DNA obtained from commercial sources and these results are shown in Table 18. It is apparent that a significant amount of the barbituric acid was adsorbed to the RNA and DNA, and that it remained bound through a number of treatments. While the levels of 2-^{14}C-barbituric acid bound in this experiment were only about 0.1 of those found in the nucleic acids from B. popilliae, the conditions were different. This does not exclude the possibility of some incorporation of the label, since previous experiments indicated a linear uptake into the RNA. However, if incorporation does occur, it is so low that it is of doubtful significance.

Further evidence that barbituric acid was not specifically incorporated into RNA monoucleotides is presented in Fig. 6. It is apparent that the ^{14}C peaks are not aligned with the ^{32}P peaks as would be expected if the 2-^{14}C-barbituric acid had been incorporated into the RNA mononucleotides. Moreover, since ^{14}C peaks are present which appear closely related to those present in uninoculated S-5 media after several days storage, and since this experiment was conducted with cells harvested from TYG

TABLE 18.--Adsorption of barbituric acid to RNA and DNA.

Sample	μmoles of barbituric acid per mg
RNA[a]	1.2×10^{-3}
DNA[a]	3.9×10^{-3}
RNA[b]	3.0×10^{-3}
DNA[b]	6.0×10^{-3}

[a]Two mg of yeast RNA was incubated with and without 2 mg of DNA in 2 ml of 0.2% phosphate buffer (pH 7.3) containing approximately 5.6×10^4 cpm/μmole of 2-^{14}C-barbituric acid. The samples were precipitated by the addition of an equal volume of cold 10% TCA and washed twice with 2 ml of cold 5% TCA. The samples containing both RNA and DNA were treated with 0.3M KOH to hydrolyze the RNA followed by the addition of an equal volume of cold 50% TCA to precipitate the DNA. The DNA was washed as described above and the precipitate collected on membrane filters with the aid of 3 washes of 2 ml of cold 0.01M HCl. The filters were glued to planchets and counted in the gas-flow counter.

[b]The experiment was carried out identically as described above with the exception that the samples were suspended in complete S-5 medium containing the radioactive barbituric acid. Both the phosphate buffer and the S-5 medium were sterilized before use.

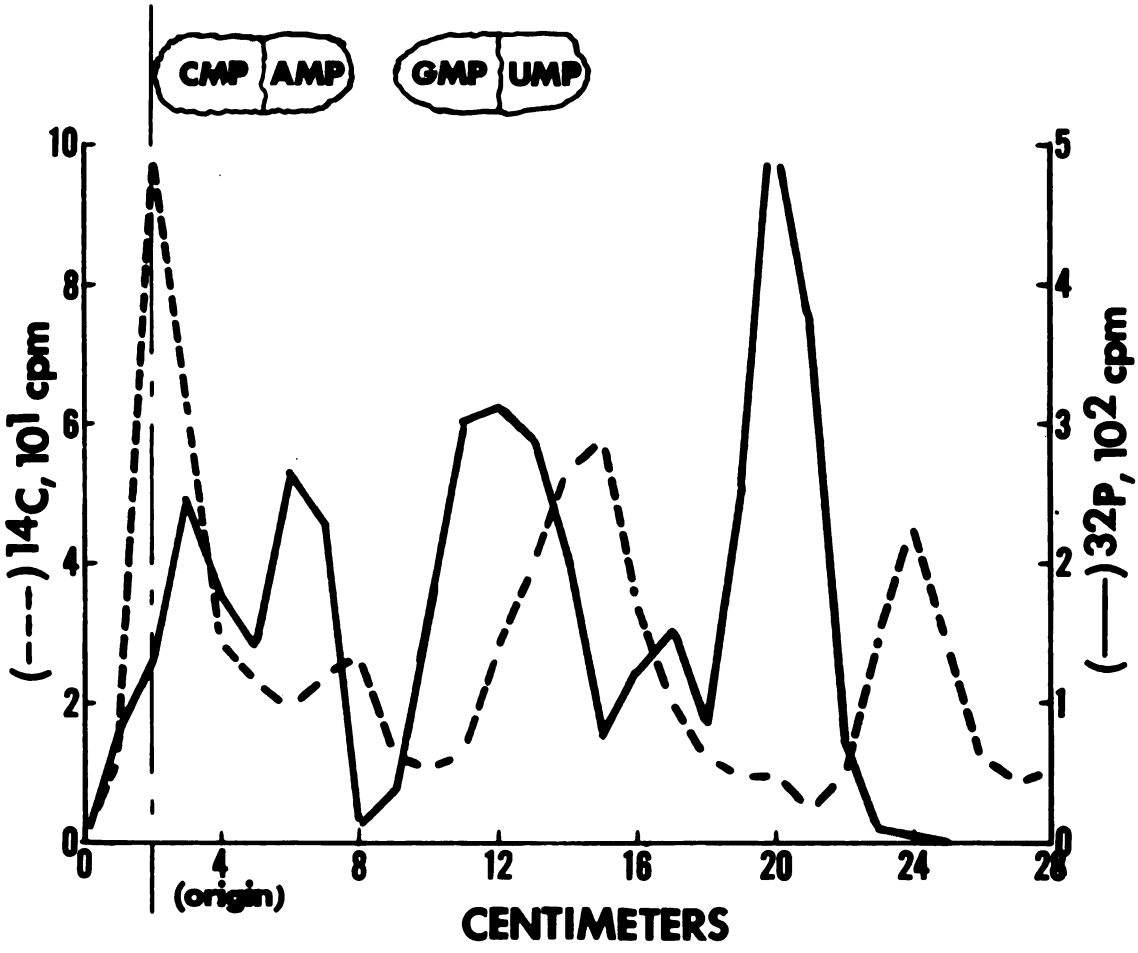


Figure 6.--Electrophoretic separation of ^{32}P- and ^{14}C- labelled RNA mononucleotides. The remaining suspensions of the control flasks of the experiment described under Table 13 were fractionated as outlined in Scheme 1 in the Experimental Methods to obtain the RNA mononucleotides. 20 µl aliquots of the labelled mononucleotides were electrophoresed at the same time on the same paper with mononucleotide standards as described in the Experimental Methods. The u/v absorbing areas were marked as shown above, and 1 cm strips were cut and placed in glass scintillation vials for the determination of radioactivity by liquid scintillation counting after the addition of 5 ml of toluene-base scintillation fluid (PPO, 4 g; POPOP, 50 mg; toluene to 1 liter).

cultures in S-5 media of unknown storage age, it appears that not only barbituric acid but the components represented by the other two peaks may be capable of binding to RNA even though they separate from the mononucleotides following alkaline hydrolysis and electrophoresis. The one ^{14}C peak at 24 cm does not correspond to any of those observed after storage of uninoculated media containing barbituric acid. This may or may not correspond to the "X" component noted earlier. In any event, it is obvious that this does not represent a mononucleotide, since no ^{32}P peak corresponded to it. These results indicate that although barbituric acid may adsorb to RNA, it does not adsorb to RNA mononucleotides.

Effect of barbituric acid on RNA and DNA synthesis.--Since only a very small amount of isotope from labelled barbituric acid was associated with the nucleic acids and this appeared to be uniformly distributed, it was apparent that cells could synthesize the required nucleotides in synthetic medium without barbiturate. Thus, this did not appear to be the major role of barbituric acid in the nutrition of B. popilliae. However, this did not exclude the possibility that barbiturate may be controlling the rate of synthesis of these macromolecules. This was the next possibility investigated.

The results shown (Fig. 7) indicated that barbituric acid did result in a strong stimulation of both RNA and DNA synthesis (29.1% and 78.8% respectively after 2 hr

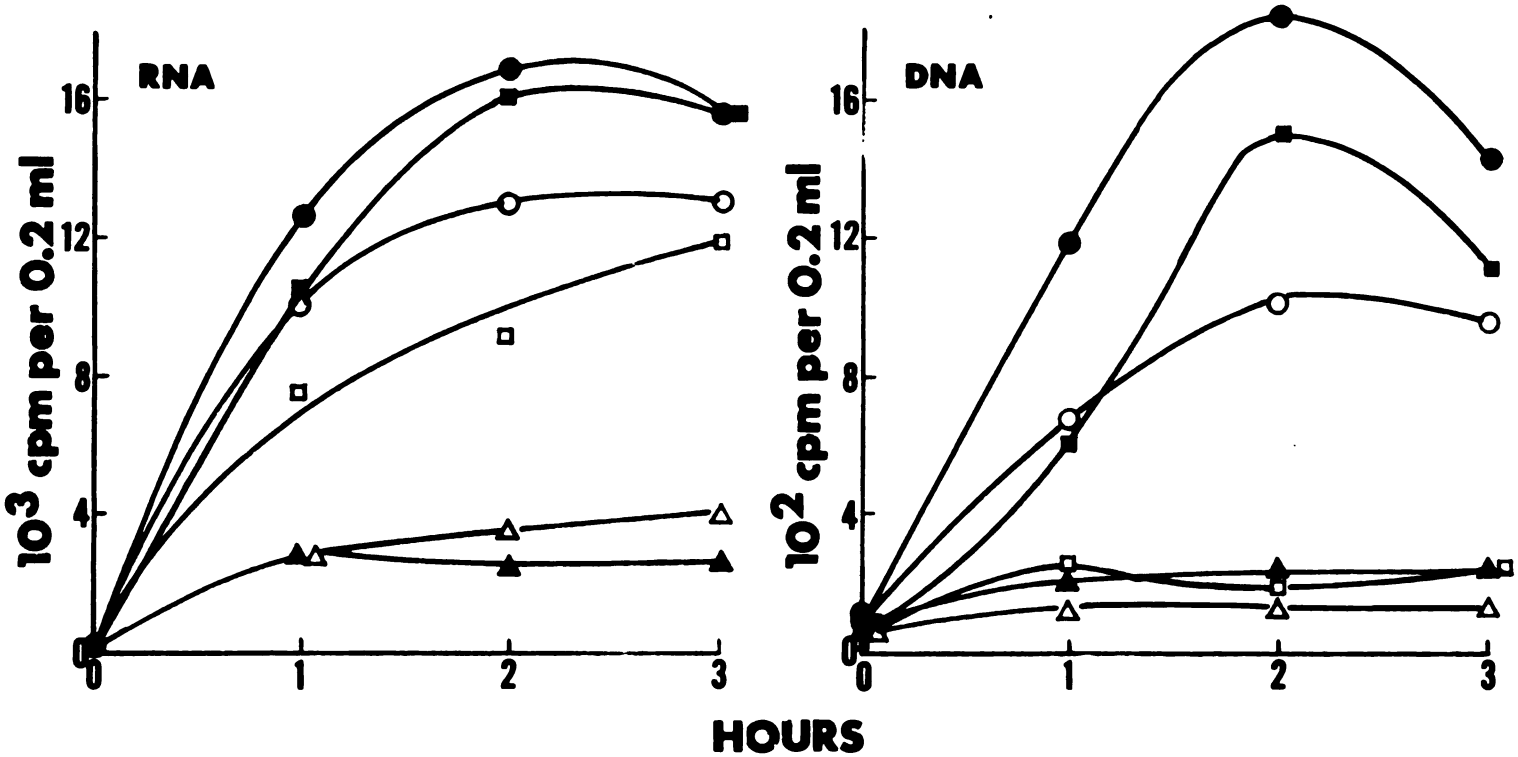


Figure 7.--Effect of barbituric acid on RNA and DNA synthesis by cells of strain NRRL B-2309M harvested from 11 hr TYG cultures. The cells were washed in BS medium with 1% glucose and with and without 0.1% barbituric acid respectively, and inoculated into the same medium containing ^{32}P at a final specific activity of 1.79×10^7 cpm/μmole in a total volume of 15 ml. 0.2 ml aliquots were removed and treated as described in the Experimental Methods. The dry weight of cells present per ml of reaction mixture was 0.52 mg. (●,o) with and without barbituric acid respectively; (▲,Δ) with and without barbituric acid respectively, but with 2 μg/ml of actinomycin D; (■,□) with and without barbituric acid respectively, but with 1 μg/ml of mitomycin C.

incubation). Actinomycin D but not mitomycin C had a marked inhibitory effect on RNA synthesis. Actinomycin D is known to inhibit selectively DNA-dependent RNA synthesis (43). The observed inhibition of DNA synthesis by this antibiotic may be due to the high concentration used. On the other hand, mitomycin C had little effect on RNA synthesis in keeping with its known property of inhibiting DNA synthesis (42). This antibiotic had an inhibitory effect on DNA synthesis in the absence of barbiturate but was not as effective in its presence. However, there was considerable variation in the results obtained from different experiments with the antibiotics. As shown in Table 19, actinomycin D had much less effect on DNA synthesis, and barbituric acid did not reverse the inhibition of DNA synthesis by mitomycin C to nearly the same extent as in the previous experiment.

The culture age at cell harvest did not have a pronounced influence on the effect of barbituric acid on the synthesis of RNA and DNA (Fig. 8). A pronounced stimulation of synthesis was observed with cells harvested from TYG cultures after 3, 6, 9, and 12 hr, and then resuspended in synthetic medium. However, the total synthesis of nucleic acids was much less from 9 and 12 hr cultures than from the 3 and 6 hr cultures. For example, the cells from 12 hr cultures synthesized 55.7% and 87.2% less RNA and DNA per mg dry weight of cells in the presence of barbiturate than did those from 6 hr cultures. The levels

TABLE 19.--Effect of antibiotics on RNA and DNA synthesis.[a]

Antibiotic	Medium	Percent inhibition	
		RNA	DNA
Actinomycin D	With barbituric acid	50.7	38.6
	Without barbituric acid	50.0	40.8
Mitomycin C	With barbituric acid	0	65.9
	Without barbituric acid	23.7	82.5

[a]The procedure was conducted on cells harvested from 6 hr TYG cultures. The cells were resuspended in BS medium with 1% glucose and with and without 0.1% barbituric acid respectively, containing 7.8 x 10^6 cpm/μmole of ^{32}P in a total volume of 15 ml. After 2 hr incubation, 0.3 ml aliquots were removed and treated as described in the Experimental Methods. Actinomycin D and mitomycin C were present in the medium at final concentrations of 0.8 and 1.0 μg/ml respectively. The flasks contained 0.77 mg dry weight of cells per ml.

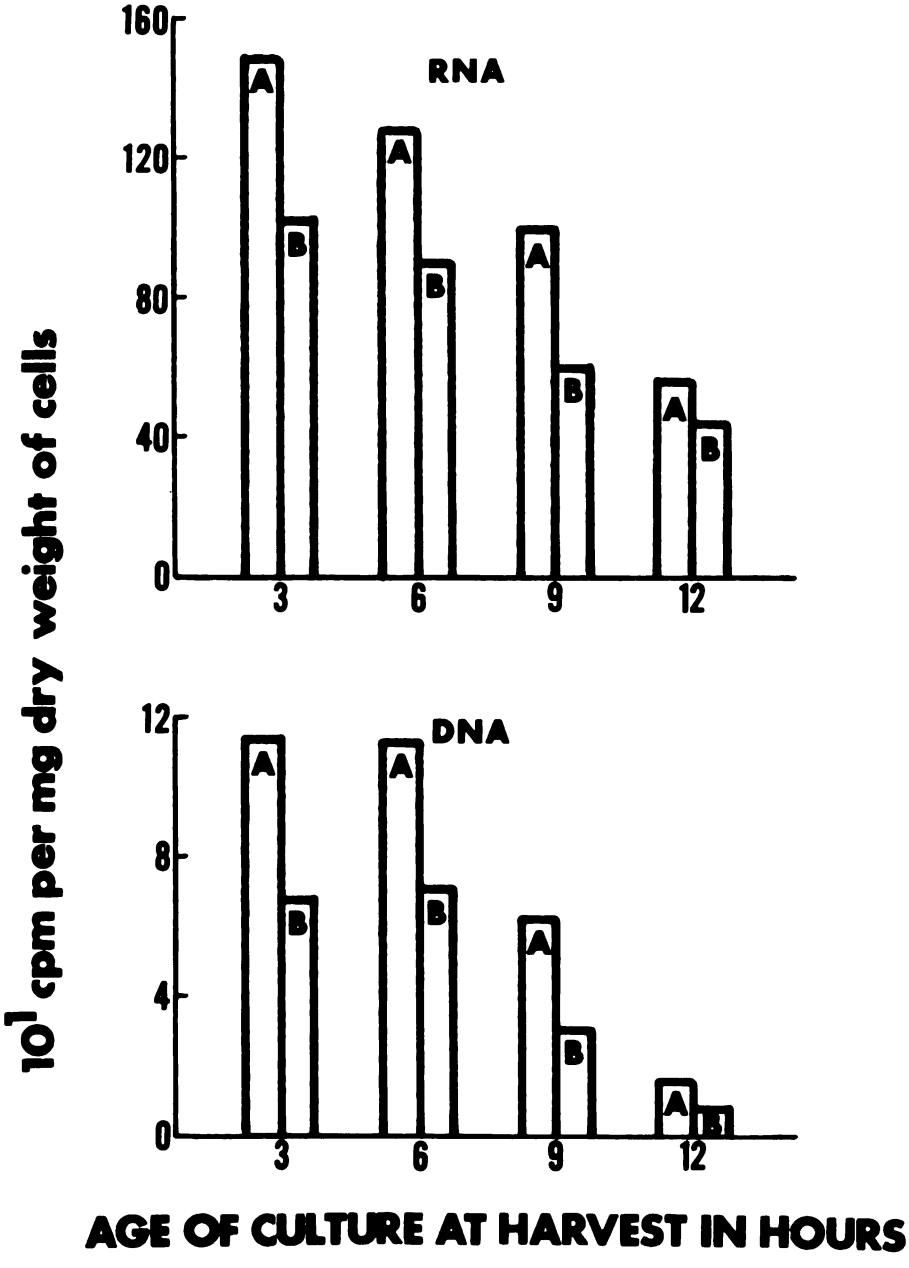


Figure 8.--Effect of age of cells on the stimulation of RNA and DNA synthesis by barbituric acid. Cells were harvested from TYG cultures and resuspended in BS medium with 1% glucose and with and without 0.1% barbituric acid respectively, and containing ^{32}P as described under Table 19. A, with barbituric acid; B, without barbituric acid.

of ^{32}P labelled mononucleotides in the cold TCA-soluble fractions of cells from 6 hr cultures was not affected by barbituric acid. The comparative levels of ^{32}P in the pool mononucleotides were 3201 and 3573 cpm in the presence and absence of barbiturate respectively.

The effect of other nucleic acid bases and barbital on RNA and DNA synthesis is shown in Table 20. Uracil and orotic acid at 0.1% levels stimulated RNA synthesis to the same extent as barbituric acid and were nearly as effective as barbituric acid for DNA synthesis. Barbital was almost as effective as the pyrimidines listed above. However, this was not apparently a replacement, since the stimulation of ^{32}P incorporation by barbituric acid was even more pronounced in the presence of all four RNA bases, each at a level of 0.01%. In contrast, the four RNA bases together at the low concentration failed to show any stimulation of synthesis. These results are of particular interest since earlier experiments revealed that uracil and orotic acid would not replace the requirement for barbituric acid for growth in S-5 medium.

Effect of barbituric acid on protein synthesis.-- Barbituric acid appeared to have an effect on protein synthesis similar to that on nucleic acid synthesis (Fig. 9). The presence of the pyrimidine resulted in approximately a 4 X greater incorporation of U-^{14}C-glutamic acid into protein after 2 hr incubation.

TABLE 20.--Effect of other pyrimidines and nucleic acid bases on nucleic acid synthesis.[a]

Medium	RNA		DNA	
	CPM	Percent of +BA	CPM	Percent of +BA
+BA	9968	--	716	--
-BA	3275	32.9	174	24.3
+U	10504	105.4	560	78.2
+O	10360	103.9	592	82.7
+B'al	8948	89.8	420	58.7
+U, C, G, A, -BA	15431	39.7	171	23.9
+U, C, G, A, +BA	15431	154.8	906	126.5

[a]Cells of strain NRRL B-2309M were harvested from 6 hr TYG cultures and resuspended in BS medium containing 1% glucose and ^{32}P at a specific activity of 1.33×10^7 cpm/µmole. The pyrimidines were present singly at a concentration of 0.1%. The flasks contained mixtures of nucleic acid bases each at an approximate concentration of 0.01%. Barbituric acid was present where indicated at a level of 0.1%. One-half ml aliquots were removed after 2 hr incubation and treated as described in the Experimental Methods. The symbols used represent the following: Ba, barbituric acid; U, uracil; O, orotic acid; B'al, barbital; C, cytosine; G, guanine; A, adenine. The dry weight of cells present per flask was 0.17 mg/ml.

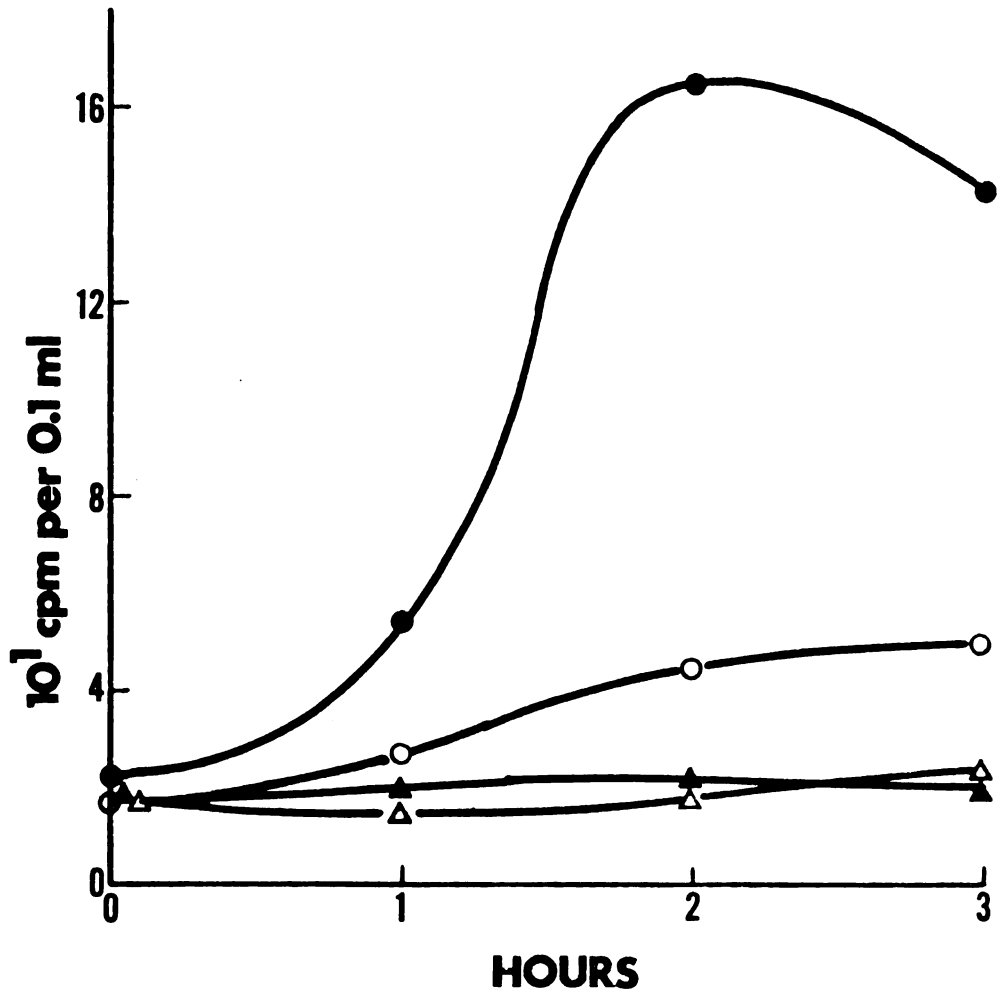


Figure 9.--Effect of barbituric acid on protein synthesis. Cells of strain NRRL B-2309M were harvested from 14 hr TYG cultures, washed in BS medium with 1% glucose and with and without 0.1% barbituric acid respectively, and resuspended in the same medium containing 2.06 x 10^8 cpm/µmole of U-^{14}C-L-glutamic acid. 0.1 ml aliquots were removed and treated as described in the Experimental Methods. (●,○) with and without barbituric acid respectively; (▲,△) with and without barbituric acid respectively, but with 100 µg/ml of chloramphenicol.

Chloramphenicol completely inhibited protein synthesis over the duration of the experiment.

As with the corresponding nucleic acid experiment, the culture age at harvest had a dramatic effect on the rate of incorporation of an amino acid into protein. At 2 hr, the proline uptake by the cells harvested from 14 hr cultures was 84.9% and 91.5% less than that observed with cells harvested from 6 hr cultures in the presence and absence of barbituric acid respectively (Fig. 10). However, barbituric acid appeared to have a similar stimulatory effect on amino acid incorporation irrespective of the age of the culture at harvest, since the proline uptake by cells harvested from 6 and 14 hr cultures was 54.7% and 62.5% greater respectively in the presence of the pyrimidine than in its absence.

Earlier experiments indicated that nucleic acid synthesis occurred to about the same extent in the presence of other pyrimidines as in the presence of barbituric acid. The results of a similar experiment carried out on protein synthesis are shown in Table 21. Since the stimulation of protein synthesis in this experiment by barbituric acid was not very pronounced, the significance of the effect of other pyrimidines and nucleic acid bases on protein synthesis is also questionable. However, none of the compounds resulted in ^{14}C-L-proline incorporation to the same extent as barbituric acid. Also, the addition of barbituric acid along

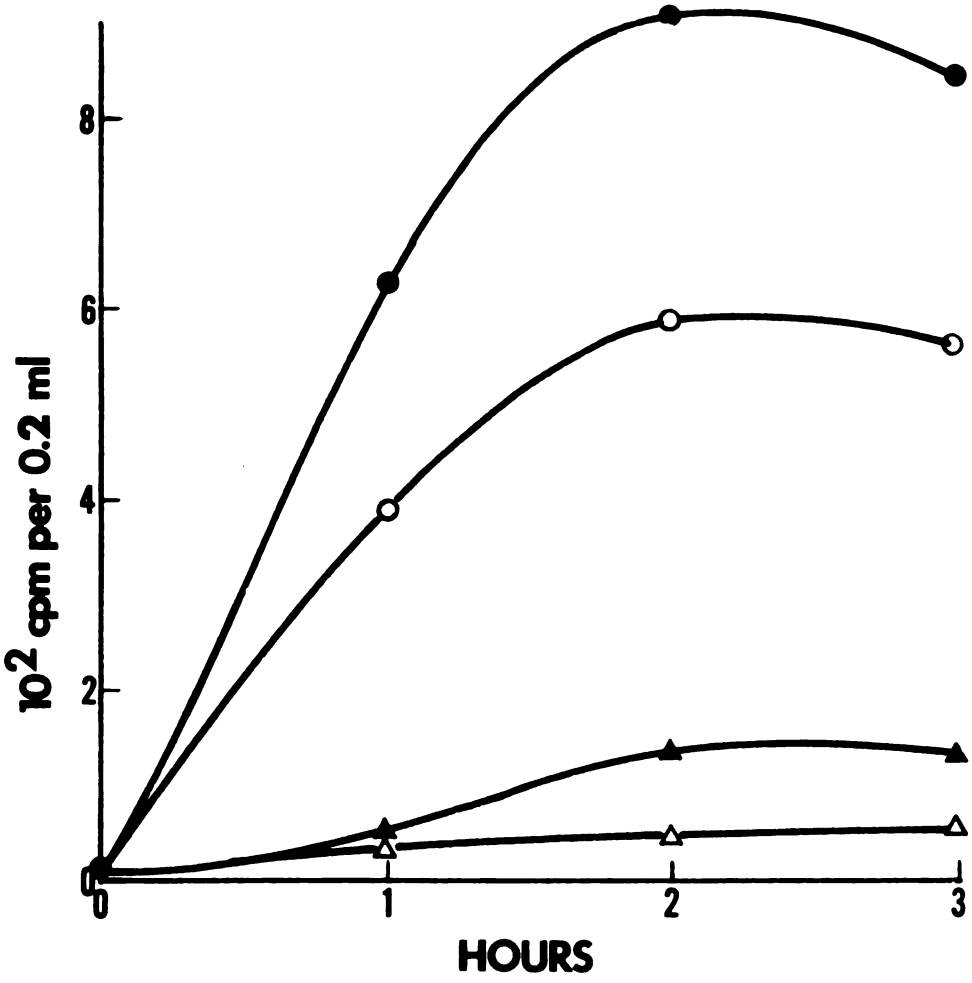


Figure 10.--Effect of culture age at time of harvest on the stimulation of protein synthesis by barbituric acid. Cells of strain NRRL B-2309M were harvested from 6 and 14 hr TYG cultures, washed in BS medium with 1% glucose and with and without 0.1% barbituric acid respectively, and resuspended in the same medium containing 5-^{14}C-DL-proline at a final specific activity of 4.79×10^5 cpm/μmole. 0.2 ml aliquots were removed and treated as described in the Experimental Methods. The flasks contained 1.5 and 2.3 mg dry weight of cells per ml for the cells harvested from 6 and 14 hr TYG cultures respectively. The radioactivity determinations obtained for the cells harvested from 14 hr cultures were divided by 1.53 so that the counts would be comparable on a dry weight basis. (●,o) with and without barbituric acid respectively, but with cells harvested from 6 hr cultures; (▲,Δ) with and without barbituric acid respectively, but with cells harvested from 14 hr cultures.

TABLE 21.--Effect of other pyrimidines and nucleic acid bases on protein synthesis.[a]

Medium	CPM	Percent of +BA
+BA	544	--
-BA	454	83.5
+U	393	72.2
+O	394	72.4
+B'al	472	86.8
+U, C, G, A, -BA	483	88.8
+U, C, G, A, +BA	661	121.5
		Percent inhibition
+BA, +actinomycin D	16	97.1
-BA, +actinomycin D	13	97.1
+BA, +mitomycin C	498	8.5
-BA, +mitomycin C	422	7.0

[a]Cells of strain NRRL B-2309M were harvested from 6 hr TYG cultures, and resuspended in BS medium as described under Table 20. The symbols used and the preparation of the media are as described under Table 20. 5-^{14}C-L-Proline was added to a specific activity of 7.58×10^7 cpm/µmole. The experiment was conducted as described under Fig. 9 with the exceptions that the flasks contained 0.48 mg dry weight of cells per ml, and 1.0 ml aliquots were removed after 2 hr incubation. Actinomycin D and mitomycin C were present where indicated at final concentrations of 0.8 and 1.0 µg/ml respectively.

with low levels of the normal bases of RNA resulted in a pronounced stimulation of isotope incorporation into protein. The protein synthesis observed was completely inhibited in the presence of actinomycin D but not in the presence of mitomycin C, which is compatible with the known effects of these antibiotics.

DISCUSSION

The evidence presented in this study confirms the previous findings (82) that barbituric acid is required for growth of B. popilliae in a synthetic medium and that this requirement is not replaced by other pyrimidines. Although barbituric acid was required in the extracellular medium at a concentration of 0.1%, it was not metabolized by the cells to an appreciable extent. This was evident from the very small amount of barbiturate depletion in the growth medium.

Barbituric acid did not appear to serve as an energy source since only very small amounts were utilized by the cells. In addition, glucose is known to be the primary energy source for this organism (82), and was required in the basal synthetic medium at a level of 1% in the presence of 0.1% barbituric acid. Cells harvested from cultures in either complex or synthetic media were incapable of oxidizing the pyrimidine to a detectable extent. It does not appear likely that the barbituric acid was degraded to malonic acid and urea as is known to occur in some bacteria (3,51), since urea was not produced from barbiturate by cells harvested from complex media. These data, alone, were not very conclusive, since it could be argued that the enzymes may be inducible. However, cells grown in complex media responded promptly to the presence of barbituric acid

as indicated by an increase in the rates of nucleic acid and protein synthesis. Catabolism through a reductive pathway as proposed for uracil fermentation by Clostridium uracilicum (10) is not very likely, since the reaction catalyzed by uracil-thymine oxidase apparently favors the formation of barbituric acid, and there was not a preferential incorporation of ^{14}C from 2-^{14}C-barbituric acid into the UMP of the cells.

The absence of a significant effect of barbituric acid on glucose oxidation or on the RQ indicates that the pyrimidine did not affect energy generation from glucose as substrate. In addition, the evidence presented in these studies does not suggest that barbiturate was effective in acting as an alternate electron acceptor from flavin, since such an effect would have resulted in reduced H_2O_2 production. Failure to inhibit H_2O_2 generation and NADH oxidation in the presence of azide showed that the pyrimidine did not interfere with the transfer of electrons from flavin to oxygen.

With regard to the inhibitory effect of barbituric acid on the electron transport system, barbituric acid is quite different from the barbiturate derivatives reported to be effective in electron transport inhibition and pharmacological activity. The effect of barbiturate derivatives on electron transport may be due to interaction with enzyme-bound flavins (29). Pharmacologically important barbiturates are thought to inhibit oxidative metabolism

through inhibition of electron transport, and it has been suggested that such interference may be the result of slight conformational changes in a membrane due to the hydrophobic bonding of the drug followed by disruption of electron flow (33). A great deal of work has indicated that, in general, there is a direct correlation between the lipophilic character of the barbiturate substituents and the hypnotic activity of the drug (22,34). Although the double substitution by lipophilic sidechains may be responsible for the mobility of the derivative to the site of action, the double substitution also results in an alteration of the dissociation property of the pyrimidine which may be significant in the effect on flavoenzymes. Kyogoku, Lord, and Rich (49) have reported that barbiturate derivatives are able to form more extensive hydrogen bonds with adenine derivatives than either thymine or uracil derivatives. The reason for this property may be the availability of more -NH-CO- sites for the formation of cyclic dimers through hydrogen bonding coupled with the fact that the pKs of the barbiturate derivatives are lower than those of the uracil or thymine derivatives. According to Doran (22) and Fox and Shugar (26), the dissociation property of doubly substituted barbiturates is due to lactam-lactim tautomerization as is that of the pyrimidines found in nucleic acids. However, the dissociation of unsubstituted barbituric acid, which is a stronger acid than any of the barbiturate derivatives

or the pyrimidines found in nucleic acids, is due to keto-enol tautomerization involving the methylene group and the pK of the dissociation involving the imide hydrogen is higher than that of the normal nucleic acid bases. Hence, the inactivity of barbituric acid as a drug and the absence of reported effects on electron transport may be due to the lack of lipophilic side chains resulting in a lower partition coefficient between nonpolar and polar solvents as well as to its unique dissociation properties.

The effect of barbituric acid on cell permeability remains unknown since a number of attempts to measure the amino acid pool of cells resuspended in the presence and absence of barbituric acid failed. However, there was no significant difference in RNA leakage from cells suspended in phosphate buffer with and without barbiturate; nor was there any effect of barbituric acid on the viability of cells suspended in buffer. In contrast, cells suspended in water leaked RNA rapidly, and there was a dramatic loss of viability.

The results of many experiments demonstrated that a small but significant amount of the isotope from 2-^{14}C-barbituric acid was found associated with the nucleic acids from B. popilliae. There was not a general distribution of the label in cell components as evidenced by the absence of label in the protein fraction. There was no evidence to suggest that the pyrimidine was degraded prior to incorporation. Thus, there was no detectable oxidation of

barbiturate, nor was there any detectable production of urea from it. The ^{14}C from 2-^{14}C-barbituric acid was found to be uniformly distributed in the 4s, 16s, 23s fractions of the RNA; thus, there was no specificity for a particular RNA. Approximately 3 x 10^{-2} μatoms of ^{14}C from 2-^{14}C-barbituric acid were consistently found in the RNA fraction. However, control experiments indicated that at least as much as 10% of this may occur by nonspecific adsorption; and no significant amount of the label was found associated with mononucleotides after hydrolysis of RNA. Thus, it appears most likely that the association of barbiturate with RNA results from binding rather than incorporation.

The results of experiments presented in this study demonstrated that barbituric acid resulted in a strong and consistent stimulation of nucleic acid and protein synthesis. The stimulatory effect of barbituric acid is unique among the pyrimidine analogues, since other analogues result in inhibition of macromolecular synthesis. 6-Azauracil is not incorporated into RNA but results in the inhibition of RNA and protein synthesis in Saccharomyces carlsbergensis (19) and in the depression of orotidylic acid decarboxylase activity in human diploid cells (65). The activity of this enzyme has also been shown to be inhibited by 5-hydroxyuracil although this compound is incorporated into RNA in very small amounts (74). On the other hand, 5-fluorouracil is incorporated into RNA and may replace as much as 70% of the uracil (19).

Although this analogue is known to inhibit the activity of thymidylate synthetase (14), its inhibitory effect is thought to be due to the formation of unstable RNA and the accumulation of immature ribosomal particles (1,19,47). The transfer-RNA of E. coli containing 5-fluorouracil was found to be unaltered in its ability to accept amino acids, to bind to ribosomes, and to transfer amino acids to polypeptides (53).

The stimulatory effect of barbituric acid on RNA, DNA, and protein synthesis in B. popilliae may be due to a stabilization of the intracellular complexes involved in the biosynthesis of macromolecules or to the activation of other metabolic pathways essential for the cell. As reported earlier, barbiturates have been found to result in elevated levels of alanine racemase activity (31) and of the enzymes involved in pyrimidine biosynthesis in E. coli (5) as well as inhibition of orotic acid uptake into the pool of B. cereus (55). However, there may be a closer parallel between the effect of barbituric acid with B. popilliae cells and the effect of barbiturate derivatives observed with mammalian systems. Although barbiturates are known to result in an increase in the synthesis of enzymes associated with drug metabolism (60,66), a stimulation of the production of ascorbic acid through the glucuronic acid pathway has also been observed (17). Phenobarbital administration results in the stimulation of DNA-dependent RNA polymerase activity in rat liver

(28); and microsomes isolated from phenobarbital-treated rats were more active in poly-U-directed phenylalanine incorporation into polypeptides (45). The effect by phenobarbital is thought to be due to an increase in the number of binding sites for the m-RNA since the drug results in a greater fraction of the isolated ribosomes being attached to the endoplasmic reticulum (44). Although bacterial ribosomes may not be associated with subcellular structures, the stimulatory effect by barbituric acid may be due to a stabilization of the complexes (polysomes) involved in macromolecular synthesis. The fact that this compound binds tightly to nucleic acids indicates that it may indeed function in such a manner.

In the course of these studies, a number of non-successful attempts were made in developing a medium which would support minimal growth of the organism without barbiturate, but in which the addition of barbituric acid would result in stimulation. Such a medium would be extremely useful for further studies. Experiments could be carried out on comparisons of enzyme activities and metabolite levels associated with various metabolic pathways, on <u>in vitro</u> amino synthetase activities, transfer-RNA accepting ability, and RNA and polysomal stability with cells harvested from cultures grown in the presence and absence of barbituric acid. It is believed that the availability of such cells for comparative experiments

would greatly aid in the elucidation of the actual mechanism by which barbiturate stimulates the synthesis of nucleic acids and proteins in B. popilliae cells.

SUMMARY

These results have confirmed previous findings (18) that barbituric acid is required for growth of B. popilliae in a synthetic medium and that this requirement is not replaced by other pyrimidines. While a high concentration of barbituric acid (0.1%) was required for maximum growth in a synthetic medium, only a very small amount (∿2%) of that added disappeared from the medium during growth.

The results of these studies effectively eliminated a number of possible effects of barbituric acid on B. popilliae cells. Barbiturate did not constitute a significant energy source since cells neither oxidized barbiturate nor hydrolyzed it to malonic acid and urea. No quantitative or qualitative effect on the energy generation from glucose was indicated. Neither the rate of oxidation of glucose nor the respiratory quotient of glucose oxidation were affected. Similarly, barbiturates failed to influence the accumulation of H_2O_2 by cell extracts, and had no effect on the rate of NADH oxidation. Finally, there was no significant effect of this pyrimidine on the leakage of RNA from cells or on the retention of cell viability. Experiments designed to determine possible effects on the accumulation of exogenous amino acids into the pool were not conclusive, but no differences were observed.

Although experiments conducted with 2-^{14}C-barbituric acid indicated that there was a small but significant amount of ^{14}C associated with both RNA and DNA in B. popilliae cells, a general distribution of the isotope among cell components did not occur as evidenced by the absence of ^{14}C incorporation into protein. The ^{14}C was uniformly distributed among the 4s, 16s, and 23s RNA fractions at a concentration of approximately 3 x 10^{-2} μatoms per mg of RNA. However, control experiments indicated that at least 10% of this may occur by nonspecific adsorption to the nucleic acids; and no significant amount of the ^{14}C was associated with the mononucleotides following hydrolysis of the RNA. Thus, it is believed that intact barbituric acid is tightly bound to the nucleic acids within the cell.

A strong and consistent stimulation of nucleic acid and protein synthesis by barbituric acid was observed. Although other pyrimidines were also found to be capable of stimulating nucleic acid synthesis, the stimulatory effect of barbituric acid was found to be most pronounced in the presence of low concentrations (0.01%) of the normal pyrimidine and purine bases. It is proposed that the stimulatory effect of barbituric acid on the growth of B. popilliae in synthetic medium may be due to a stabilization of the complexes (polysomes) involved in macromolecular synthesis.

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