AN ABSTRACT OF THE THESIS OF

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Dr. D. K. Fraser

The purpose of this research was to determine if a basic protein was associated with the DNA of Bacillus subtilis W168, and to determine if there was a difference in the amount and type of protein associated with the DNA at different times during the growth cycle of the organism. The amino acid compositions of the cellular proteins at these times were also determined for comparison with any protein found associated with the DNA. The plan of approach was to grow cells for 5, 8, and 11 hours, and to extract the DNA complexes from these cells according to a modification of the procedure described by Marmur. The "DNA/protein" ratios of the DNA complexes, based upon the Lowry and diphenylamine tests, were 1:0.33, 1:0.35, and 1:0.36 for the 5, 8, and 11 hour cultures respectively. Ultraviolet absorption spectra of these DNA complexes showed that, except for the five hour culture, some material was associated with the DNA. An amino acid analysis of the DNA complex isolated from the eight

hour culture showed the amino acids glutamic acid, glycine, alanine, and methionine to be present in significant amounts. No basic amino acids were found in the sample. An unknown peak, possibly glucosamine, was also observed to be present.

The DNA complexes were treated with 3 M NaCl to dissociate any protein from the DNA. Ultraviolet absorption spectra of the DNA samples obtained after this treatment were typical of those of pure DNA, indicating that some material had been removed. The material obtained from the dissociation treatment was found to contain very little protein. This material was treated with 0.25 N HCl according to Butler and Godson's method for separating acidic and basic fractions. Very little acidic material was present. The three basic fractions were found to be similar when analyzed for total nitrogen, organic phosphorus, and amino acids. An amino acid analysis of the basic fraction obtained from the five hour culture showed the presence of trace amounts of all of the amino acids except lysine, histidine, arginine, and 1/2 cystine. The amino acids, however, were present in such low amounts as to make it uncertain that their presence was not due to contamination. An unknown peak, corresponding to the peak found in the amino acid analysis of the DNA complex isolated from the eight hour culture, was also observed to be present in this analysis.

The middle layers obtained during the extraction of the DNA

complexes were separated into acidic and basic protein fractions using 0.25 N HCl. These fractions were then hydrolyzed and analyzed for the presence of amino acids by two-dimensional paper chromatography. The basic protein fractions contained the most material, by weight, but very few amino acids were identified in these fractions. Most of the material present in these basic fractions was considered to be poly-β-hydroxybutyric acid. The amino acids found in the acidic protein fractions were basically the same for all three cultures.

Materials Associated with the DNA Fraction of *Bacillus subtilis* W168

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

MATERIALS ASSOCIATED WITH THE DNA FRACTION OF *BACILLUS SUBTILIS* W168

INTRODUCTION

The finding that deoxyribonucleic acid (DNA) is the genetic material of all living organisms has led to extensive studies on the role of this macromolecule in cellular development. A knowledge of the mechanisms which regulate cell growth and division, and of the factors which influence the structure and properties of the DNA is a prerequisite to understanding the development and differentiation of biological systems.

The bacterial chromosome is a closed ring structure consisting of a single piece of double-stranded DNA. There are considered to be two chromosomes present in the bacterial cell, but the number of chromosomes present is dependent upon the growth rate of the organism. The chromosome is attached to the cell wall-membrane complex, and replication proceeds sequentially in a semiconservative manner from the beginning to the end of the chromosome. Protein synthesis is required to initiate replication of the DNA, but once initiated, the replication cycle continues to completion even if protein synthesis is inhibited.

The role of DNA in coding for the RNA and proteins of the cell is well known, although, in some respects, not yet completely

understood. It is known that specialized cells contain different proteins, and since all of the genes are not functional at the same time, there must necessarily be some means of regulating the expression of these genes. In higher organisms and plants, this regulation is thought to be controlled, in part, by the presence of basic proteins called histones. The histones are believed to function as gene regulators in that they can prevent the expression of the genes along the DNA molecule, thereby permitting only certain genes to be read at any one time.

If histones are the sole means of turning "off" and "on" genes, then it would be reasonable to expect that histones would also be present in bacterial cells. As yet, however, basic proteins associated with the DNA of bacterial cells have not been identified. Alternatively, Jacob and Monod (1961) have proposed an "operon" model whereby gene expression is controlled in bacterial cells. It may well be that the method of genetic regulation in bacterial cells differs from that of higher organisms. If this is the case, the occurrence of histones in higher organisms may have arisen during evolution as a different type of gene repression in specialized tissue cells.

This study was undertaken to determine if a basic protein was associated with the DNA of Bacillus subtilis W168, and if there was a

difference in the amount and type of protein associated with the DNA at different times during the growth cycle of the organism.

HISTORICAL

The discovery of the biological importance of the nucleic acids and nucleoproteins in the cell has led to extensive investigation of the biochemical and biophysical properties of these macromolecules. The nucleoproteins, especially, are of interest since it has been shown that the deoxyribonucleic acid (DNA) of higher organisms and plants is always found associated with a basic protein (Bonner and Huang, 1963; Bonner, 1965; Murray, 1965). This basic protein is thought to function as a gene regulator, but the complete significance of its relationship with the DNA has yet to be determined (Stedman and Stedman, 1943; Huang and Bonner, 1962; Allfrey, Littau, and Mirsky, 1963; Bonner, 1965).

There are two classes of basic proteins: protamines and histones. Generally speaking, protamines refer to basic proteins extracted from spermatozoa, and histones refer to basic proteins extracted from the nuclei of somatic cells. Histones have a high molecular weight (> 10,000), and they consist of a mixture of proteins. They are characterized by a high content of the basic amino acids arginine and lysine, and by a lack of the amino acids tryptophan and cystine. On the basis of their molar ratio of lysine/arginine, the histones are referred to as either arginine rich, slightly lysine rich, or very lysine rich (Peacocke, 1960; Phillips, 1962). The

basic amino acid residues comprise approximately 23 to 30 percent of the total amino acid residues present in histones (Shooter, Davison, and Butler, 1954).

By carefully extracting calf thymus glands at very low concentration and very low ionic strength, Zubay and Doty (1959) obtained gel-free solutions of DNP particles having a molecular weight of 18.5 million. Dissociation of the DNP particles gave DNA molecules having a molecular weight of eight million. This was indicative of a DNP particle consisting essentially of one DNA molecule and an equal amount of protein.

Histones can be extracted from deoxyribonucleoproteins in a variety of ways, but essentially two standard procedures are commonly used. They can be extracted with dilute acid from whole cells, isolated nuclei, or precipitated DNP; or, alternatively, the DNP can be dissociated in solutions of high salt concentration and the histone then separated from the DNA (Bernstein and Mazia, 1953; Crampton, Lipshitz, and Chargaff, 1954; Davison, Conway, and Butler, 1954; Smillie, Marko, and Butler, 1955; Bayley, Preston, and Peacocke, 1962; Bauer and Johanson, 1966). Extraction of histones by either method results in a stepwise cleavage of the histone into various fractions (Crampton, Lipshitz, and Chargaff, 1954; Lucy and Butler, 1955; Smillie, Butler, and Smith, 1958; Neelin and Butler, 1959; Johns and Butler, 1964; Murray, 1965).

Johns (1966) separated the histones isolated from calf thymus into five different fractions based on their molar ratios of lysine/arginine. Each fraction represented 20 percent of the total histones of calf thymus.

Experiments so far have not provided evidence for any specificity of interaction between specific histone fractions and specific parts of the DNA (Phillips, 1960; Johns and Butler, 1964; Johns, 1966). There is, however, a marked difference in the way in which the various histone fractions are combined with the whole of the DNA. Studies using electrophoresis and ion-exchange chromatography have shown that the very lysine-rich histones are the most easily removed from nucleoproteins, and that the nucleoprotein contains some molecules in which DNA with a high guanine and cytosine content is extracted with a histone containing a high proportion of lysine; whereas DNA with a high adenine and thymine content is extracted with a histone rich in arginine. These differences could not be accounted for by differences in the net positive charge of the various fractions, but they could possibly be accounted for by the number of basic amino acids sterically available for combination with the phosphate groups of the DNA due to the shape of the histone molecule.

As well as being associated with histone, DNA from animal cells is also found associated with a residual protein (Thomas and

Mayer, 1949; Mirsky and Ris, 1951; Bernstein and Mazia, 1953; Monty and Dounce, 1958; Frearson and Kirby, 1964; Lesko, Kulkarni, and Emery, 1964; Wang, 1965). This protein seems to be firmly bound to the DNA since exhaustive treatment with 0.2 N HCl or 1 M NaCl, pH 3, followed by subsequent treatment with detergent will not remove the protein. This "residual protein fraction" is acidic, contains RNA and DNA in varying amounts, and, in contrast to histones, tryptophan. Wang (1965) has used radioactive labelling to show that the residual protein fractions are very active in nuclear protein synthesis which in turn is related to their RNA content.

The residual proteins are believed to be the morphological backbone of the chromosomes. Mirsky and Ris (1951), using differential staining techniques, showed that histone could be removed from the chromosomes without affecting their microscopic appearance, but if the residual protein was removed, the appearance of the chromosomes was altered considerably.

The structure of the nucleohistone molecule has not been fully elucidated. Bayley, Preston, and Peacocke (1962) have shown that the histone can be removed from the DNA without affecting the molecular shape of the DNP complex. This rules out a nucleoprotein structure in which the histone component forms cross-links, or in which the histone links together DNA subunits in a linear chain.

Results of X-ray diffraction patterns, electron microscopy, infrared spectroscopy, and optical rotary dispersion measurements indicate that the protein is in an alpha-helical form and is evenly distributed along the DNA molecule (Wilkins, Zubay, and Wilson, 1959; Zubay and Doty, 1959). On the basis of low angle X-ray diffraction studies of DNP gels, Luzzati and Nicolaieff (1963) have suggested a nucleoprotein model consisting of parallel DNA rods with water and histones filling the spaces between the DNA molecules. Kirby (1957) and Hagen (1960) have suggested that some form of metal linkage is involved in the binding of the histone to the DNA.

Since the number of positively charged basic groups of the histone is equivalent to the number of negatively charged phosphate groups of the DNA, the structure of the nucleoprotein molecule will have to consist of an inner core of the DNA double helix with the protein distributed along the molecule in some manner which would allow the basic amino groups and the negative phosphate groups to interact. The presence of residual proteins associated with the DNA will also have to be taken into account in the over-all structure.

In contrast to animal cells, the presence of a basic protein clearly associated with the DNA of bacterial cells has not been demonstrated (Chargaff and Saidel, 1949; Tsumita and Chargaff, 1958; Butler and Godson, 1963). Zubay and Watson (1959) avoided the use of deproteinizing agents in isolating a nucleoprotein fraction from

Escherichia coli B-2. They estimated the amino acids in a hydrolysate of the intact nucleoprotein, but it was not possible to tell whether the protein at neutral pH was acidic or basic. Wilkins and Zubay (1959) used X-ray diffraction techniques to study the association of this protein with the DNA in E. coli. The diffraction patterns showed that the protein was not bound closely to the DNA, but that it occurred in fragments attached to the DNA at relatively few points. They calculated that the weight of protein that might be attached to the DNA bases was not more than about 20 percent that of the DNA. Their results compare with those of Bekker (1964) who found that the DNA of Pasteurella pestis was bound, if at all, to no more than 25 percent protein. This material could represent DNA that is attached to membrane fragments. Masui et al. (1962) isolated a deoxyribonucleoprotein fraction from halophilic Achromobacter sp. 101 by extracting the cells with 0.01 M sodium citrate, precipitating out the cellular proteins with $(NH_4)_2SO_4$, dialyzing the supernatant, and then precipitating the DNP in 66% ethanol. The DNP was hydrolyzed, and the amino acids were identified by two-dimensional paper chromatography followed by quantitative estimation of the amino acids by spectrophotometric means. The results indicated that a basic protein was not present. Berns and Thomas (1965) were unable to find any protein bound to the DNA of Hemophilus influenzae which would be detectable by banding in a CsCl density gradient.

Leaver and Cruft (1966) studied the distribution of basic proteins in ribosomes, DNA-protein complexes, and whole cells of Staphylococcus aureus, Micrococcus lysodeikticus, Bacillus megaterium KM, and E. coli B. They found that whole cells contain between 0.1 and 1 percent basic proteins, most of which are located in the ribosomes. Attempts to isolate DNA-protein complexes from these organisms resulted in very low yields (0.1 to 0.2 percent of the total dry weight of cells). Estimations of the total DNA and of the total basic proteins in the cell established that most of the DNA was not associated with basic proteins. Ultraviolet absorption studies and starch-gel electrophoresis patterns of the basic proteins extracted from whole cells and from ribosomes gave results similar to those obtained with mammalian histones.

Cruft and Leaver (1961) reported the isolation of basic proteins from whole cells of S. aureus, but they were unable to say whether these basic proteins originated from association with DNA or with RNA. It is likely, however, that these basic proteins were derived from the ribosomal protein rather than from the DNA since ribosomes have been found to contain basic proteins (Waller and Harris, 1961; Leaver and Cruft, 1966).

Bhagavan and Atchley (1965) isolated a DNP complex from Bacillus subtilis SB19 by extracting protoplasts with 1 M NaCl, pH 8, and precipitating the DNP with 0.01 M $MgCl_2$. This DNP

complex, representing approximately 0.2 percent of the wet weight of cells extracted, contained 43 percent DNA, 5 percent RNA, and 40 percent basic proteins. These values are somewhat higher than those reported by Leaver and Cruft (1966) who isolated DNP from B. megaterium that represented 0.1 percent of the dry weight of cells extracted, and contained 9 percent DNA, 2 percent RNA, and 10 percent basic proteins. These differences may be accounted for by the differences in procedure, but in view of the apparent absence of basic proteins associated with DNA reported here and in other work, it is possible that the DNP isolated by Bhagavan and Atchley was contaminated with ribosomes. Electrophoresis of the acid soluble protein extracted from their DNP showed the protein to be basic, and amino acid analysis revealed the absence of cystine and tryptophan, deficits which are characteristic of histones. However, the ratio of basic amino acids to acidic amino acids (0.62) was considerably less than that expected for histones (1.92).

Yoshikawa (1966) has recently reported the isolation of a DNA-protein complex from protoplasts of a thymine-requiring mutant of B. subtilis. Protoplasts were osmotically disrupted and treated with phenol in the presence of 2-mercaptoethanol (MCE), and the extract then subjected to CsCl density gradient centrifugation. The presence of two DNA peaks was observed: a normal peak at a density of 1.72, and a second peak at a density of 1.63. The presence of MCE during

the phenol extraction was essential for the isolation of this second peak. Pulse labelling experiments showed that ^{14}C-lysine and ^{3}H-thymidine were incorporated into the second peak. After treatment with pronase or with 1 M MCE, the density of the second peak shifted to 1.72. These results showed that the second peak was a DNA-protein complex and suggested that the complex was hydrogen bonded rather than ionic bonded. This DNA-protein complex could be isolated from slow growing cells with a generation time longer than three hours, but it could not be isolated from either stationary cells or from spores. Isolation of a DNA-protein complex from cells with a normal generation time was apparently not attempted. On the basis of his results, Yoshikawa suggested that a DNA-protein complex is characteristic only of the replicating chromosome and not of the resting chromosome.

METHODS AND MATERIALS

Bacterial Culture

Bacillus subtilis W168 was obtained from the culture collection of the Department of Microbiology at Oregon State University.

Media

Nutrient broth-yeast extract medium (NBY) was composed of eight grams of Difco nutrient broth and three grams of Difco yeast extract per liter.

Potato medium was prepared as follows: Diced potatos (200 grams) were boiled five minutes in one liter of water and the material was filtered through Whatman No. 1 filter paper on a Buchner funnel. Two grams of Difco yeast extract and 20 grams of N-Z Case peptone were added. The pH was adjusted to 7.2 with HCl and/or NaOH, and the solution was diluted to two liters.

Nutrient agar was composed of 8 grams of Difco nutrient broth and 15 grams of Difco agar per liter.

Preparation of Spores

One loopful of an overnight culture of the organism was inoculated into a 500 ml flask containing 100 ml of potato broth, and

the flask was incubated, with shaking, for 5 days at 37° C. The spores were centrifuged at 6,000 rpm for 10 minutes, and the pellet was washed three times with sterile distilled water. The spores were resuspended in 10 ml of sterile distilled water, and the suspension was divided into two 5 ml aliquots. The spores were then heat shocked at 65° C for one hour.

The number of viable spores in the suspensions was determined by plating appropriate dilutions on nutrient agar. The spores were stored at 4° C.

Growth Curve

A 50 ml sidearm flask containing 10 ml of NBY broth was inoculated with 4.8×10^6 spores/ml. The culture was incubated, with shaking, at 37° C. The blank consisted of 10 ml of NBY broth without spores. The optical density at 650 mμ was determined at 15 minute intervals over a period of 12 hours using a Coleman Junior spectrophotometer. The growth curve of the organism is shown in Figure 1.

Preparation of the Cultures

Five, eight, and eleven hour cultures of the organism were used in this study. One liter flasks containing 250 ml of NBY broth were inoculated with 4.8×10^6 spores/ml. The flasks were incubated

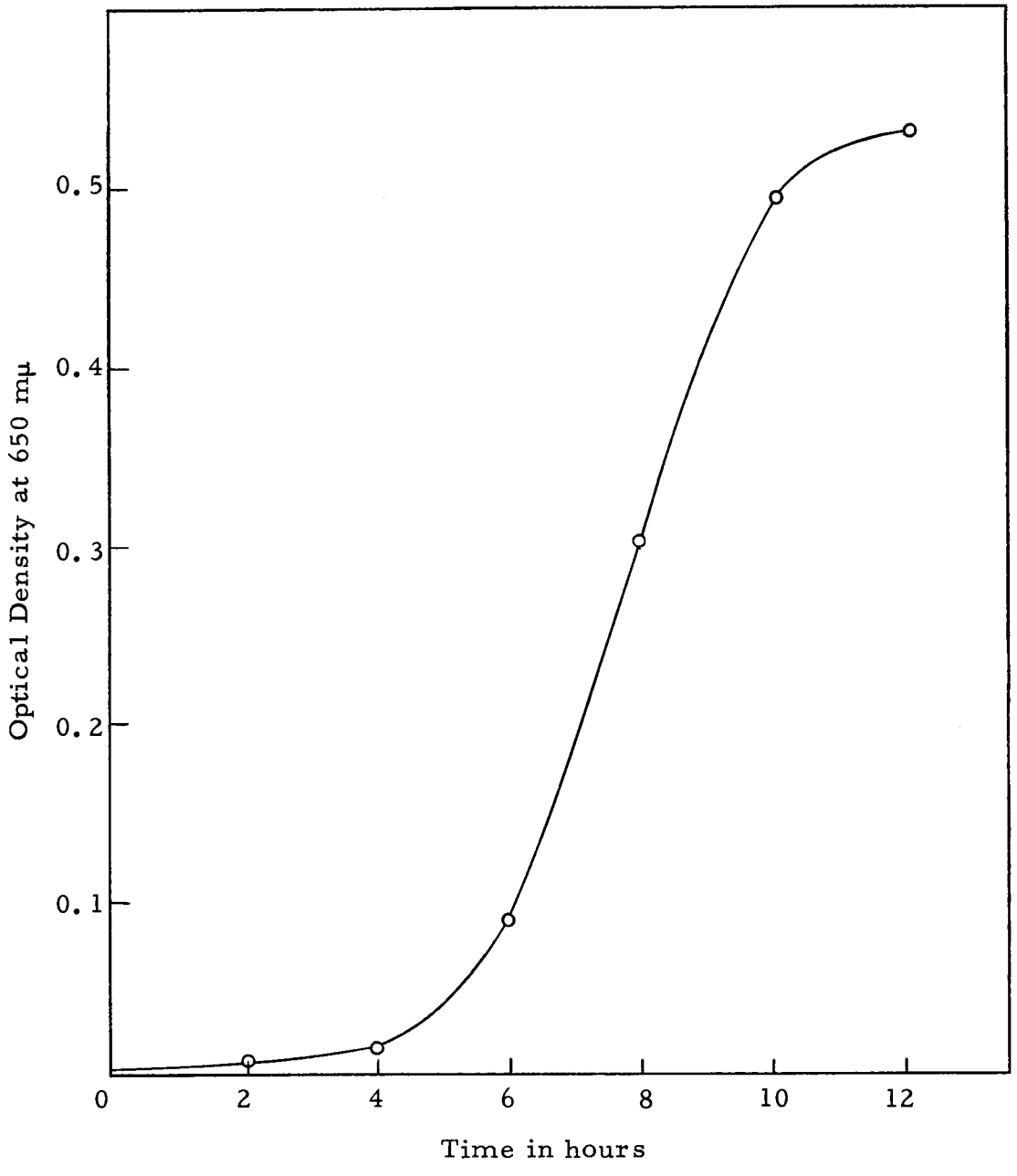

Figure 1. Growth curve of Bacillus subtilis W168.

on a rotary shaker at 37° C. At the appropriate times, the cells were harvested by centrifugation at 6,000 rpm for 10 minutes, and then washed once in saline-EDTA solution (0.15 M NaCl plus 0.1 M EDTA, pH 8). Two and a half grams (wet weight) of cells were collected from each culture. The cells were stored at -10° C until used.

Isolation of the DNA Complexes

A DNA complex is defined as the material isolated as the DNA fraction in Marmur's (1961) extraction procedure which is still combined with some unknown substances. Each of the DNA complexes was isolated as follows. The frozen cells were thawed at room temperature and washed once in saline-EDTA, pH 8. They were resuspended in 25 ml of saline-EDTA, pH 8, in a 125 ml ground glass stoppered flask. Lysozyme (40 mg/ml; 3 X crystallized preparation from egg white obtained from the California Corporation for Biochemical Research) was added to a final concentration of 1 mg/ml, and the flask was incubated, with occasional mixing, in a 37° C water bath for 45 minutes. Two milliliters of a 25% solution of sodium lauryl sulfate was added, and incubation was continued in a 60° C water bath until the suspension became clear. The flask was then cooled to room temperature and 3.9 ml of 5 M sodium perchlorate was added to the mixture, followed by the addition of an equal volume

of chloroform-isoamyl alcohol (24:1 v/v). The resulting emulsion was shaken on a rotary shaker (160 cycles/min) for 30 minutes, and then centrifuged at 8,000 rpm for 10 minutes. The aqueous top layer, containing the nucleic acids, was removed and transferred into a flask. The remaining contents of the centrifuge tube were poured into an evaporating dish. This latter mixture is referred to as the protein layer. An equal volume of chloroform-isoamyl alcohol was again added to the nucleic acid portion, and the mixture was shaken on a rotary shaker for 10 minutes and then centrifuged at 8,000 rpm for 10 minutes. The nucleic acid layer was removed as before, and the remaining contents of the centrifuge tube were poured into a second evaporating dish. Extraction of the aqueous layer with chloroform-isoamyl alcohol was carried out in this manner a total of four times. The protein layers from the second, third, and fourth extractions were combined. The nucleic acid layer from the last extraction was transferred into a large test tube and two volumes of cold 95% ethanol were gently layered over top. A glass rod was inserted into the test tube, and the DNA complex was wound up and dissolved in 9 ml of cold dilute saline-citrate solution (0.015 M NaCl - 0.0015 M Na citrate, pH 7). When the DNA complex had completely dissolved, concentrated saline-citrate (1.5 M NaCl - 0.15 M Na citrate, pH 7) was added to bring the concentration of the solution up to standard saline-citrate conditions (0.15 M NaCl -

0.015 M Na citrate, pH 7). Ribonuclease (0.2% in 0.15 M NaCl, pH 5, heated at 80° C for 10 minutes; Sigma Chemical Corporation) was added to a final concentration of 50 μg/ml, and the solution was incubated at 37° C for 30 minutes. The DNA complex was reprecipitated in 95% ethanol and dissolved in 10 ml of standard saline-citrate. An outline of the procedure is shown in Figure 2.

Dissociation of the DNA Complexes

The DNA complexes were dissociated according to a procedure described by Smillie, Marko, and Butler (1955). The three samples were adjusted to contain the same concentration of DNA in 5 ml of standard saline-citrate. The salt concentration was brought up to 3 M by the addition of 0.8329 grams of solid NaCl. The tubes were allowed to stand, with occasional mixing, at 5° C for eight hours, after which time an equal volume of cold 95% ethanol was slowly added while stirring the mixture with a glass rod. The DNA was wound up and dissolved in 5 ml of standard saline-citrate.

The ethanolic solutions, containing the material dissociated from the DNA were dialyzed against 3.75 M NaCl for 8 hours, then against running water for 12 hours, and finally against distilled water. An aliquot of each dialysate was removed for protein determination, and the remainder of the solutions were lyophilized. These samples represent the material associated with the DNA.

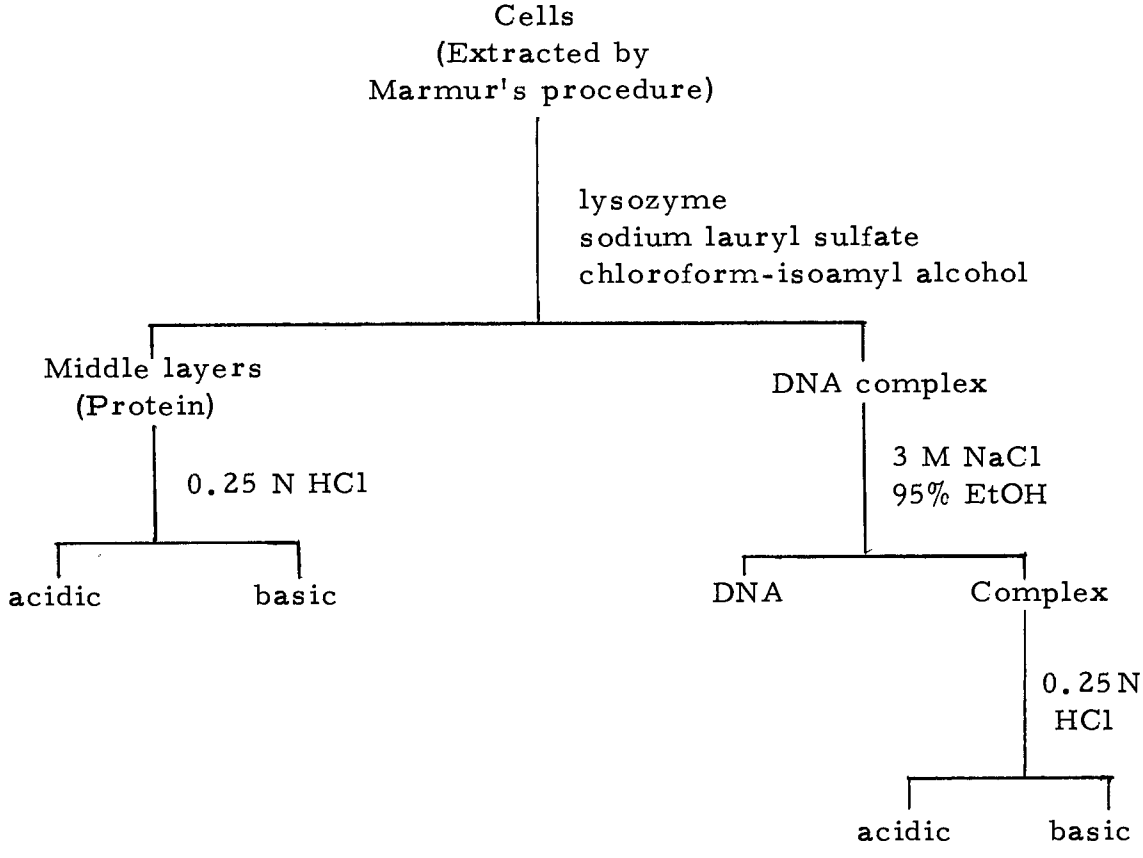

Figure 2. Fractionation of the DNA complexes.

Separation of Proteins into Acidic and Basic Fractions

The protein layers were separated into acidic and basic protein fractions according to a modification of the procedure described by Butler and Godson (1963). The protein layers resulting from the treatment with chloroform-isoamyl alcohol were evaporated to dryness in a 45° C oven. The samples were weighed and placed in 50 ml beakers. Ten milliliters of 0.25 N HCl was added, and the beakers were shaken on a rotary shaker (140 cycles/min), in the cold, for 12 hours. The samples were then centrifuged at 10,000 rpm for 20 minutes in a refrigerated Servall centrifuge, and the supernatants were dialyzed overnight in the cold against 0.25 N HCl. The pH of the dialysates was adjusted to between 8 and 9, and 0.4 ml of 6 N HCl was added to each sample. They were shaken again in the cold for four hours and then centrifuged at 10,000 rpm for 20 minutes. The supernatants were adjusted to pH 8 and lyophilized. These samples represent the basic protein fractions.

The precipitates from the above centrifugations were dissolved in 10 ml of distilled water, dialyzed overnight against running water, and lyophilized. These samples represent the acidic protein fractions.

The same procedure was used for separating the material dissociated from the DNA into acidic and basic fractions except that

the volume of reagents used was halved since the amount of material present was smaller. Instead of being lyophilized, these acidic and basic fractions were concentrated and dried by rotary evaporation in 5 ml round bottomed drying ampoules.

Chromatography

The protein samples were hydrolyzed as described by Moore and Stein (1963), and the amino acids identified by two-dimensional descending paper chromatography as described by Hausmann (1952).

Hydrolysis

Five milligrams of each sample was placed in a 5 ml round bottomed drying ampoule, and 1 ml of 6 N HCl was added to the tubes. The contents of the tubes were frozen in an acetone-dry ice bath, and the air was evacuated from the tubes. The tubes were sealed, while under vacuum, using an oxygen torch. They were then placed in a toluene bath and hydrolyzed for 21 hours, after which time the vacuum was released and the acid removed by rotary evaporation. The samples were washed with distilled water and dried by rotary evaporation. They were stored at 4° C until used. A control sample of 5 mg of vitamin-free Casamino acids (Difco) was similarly treated.

Paper Chromatography

Paper chromatograms were made of each of the samples and the Casamino acid control. The samples were dissolved in 0.5 ml of 10% isopropanol and 0.02 ml was spotted, using a lambda pipette, on 18 x 22 Whatman No. 1 chromatography paper. The papers were first exposed to a solvent consisting of sec-butanol - 3% ammonia (5:2 v/v) for 40 hours. They were then air-dried and exposed to a second solvent consisting of sec-butanol - 88% formic acid - water (15:3:2 v/v) for 12 hours. The amino acids were identified by spraying the papers with a 0.25% solution of ninhydrin in acetone and allowing the amino acid spots to develop in the dark.

The amino acids appearing on the chromatograms of the samples were identified by comparison with a similarly treated Casamino acid control.

Determination of DNA

The concentration of DNA was determined by the diphenylamine reaction as described by Burton (1955). The diphenylamine reagent was prepared by dissolving 1.5 gm of steam distilled diphenylamine (Fisher Scientific Company) in 100 ml of glacial acetic acid, and adding 1.5 ml of concentrated H_2SO_4. Just before use, 0.1 ml of aqueous acetaldehyde (16 mg/ml) was added for each 20 ml of reagent

used. The reagent was stored in the dark.

An aliquot of the sample was mixed with an equal volume of 1 N perchloric acid (PCA), and the mixture was heated at 70° C for 15 minutes. Dilutions of this solution were made with 0.5 N PCA, and one volume of sample was mixed with two volumes of the diphenylamine reagent. The tubes were incubated in a 30° C water bath for 20 hours. The optical density at 600 mμ was read in a Zeiss spectrophotometer against a blank of 0.5 N PCA that had been prepared in the same manner.

A standard curve (Figure 3) was prepared by dissolving 8 mg of purified calf thymus DNA (Sigma Chemical Company) in 20 ml of 5 mM NaOH. Five milliliters of this stock solution were mixed with 5 ml of 1 N PCA, and the solution was heated at 70° C for 15 minutes. Dilutions of this working standard were made with 0.5 N PCA to obtain concentrations of DNA ranging from 10 to 100 μg/ml. The diphenylamine reaction was carried out as described above, and the optical density at 600 mμ was plotted against the concentration of DNA.

Determination of RNA

The concentration of RNA was determined by the orcinol reaction as described by Mejbaum (1955). The orcinol reagent was prepared by dissolving 0.1 gm of ferric chloride and 0.1 gm of

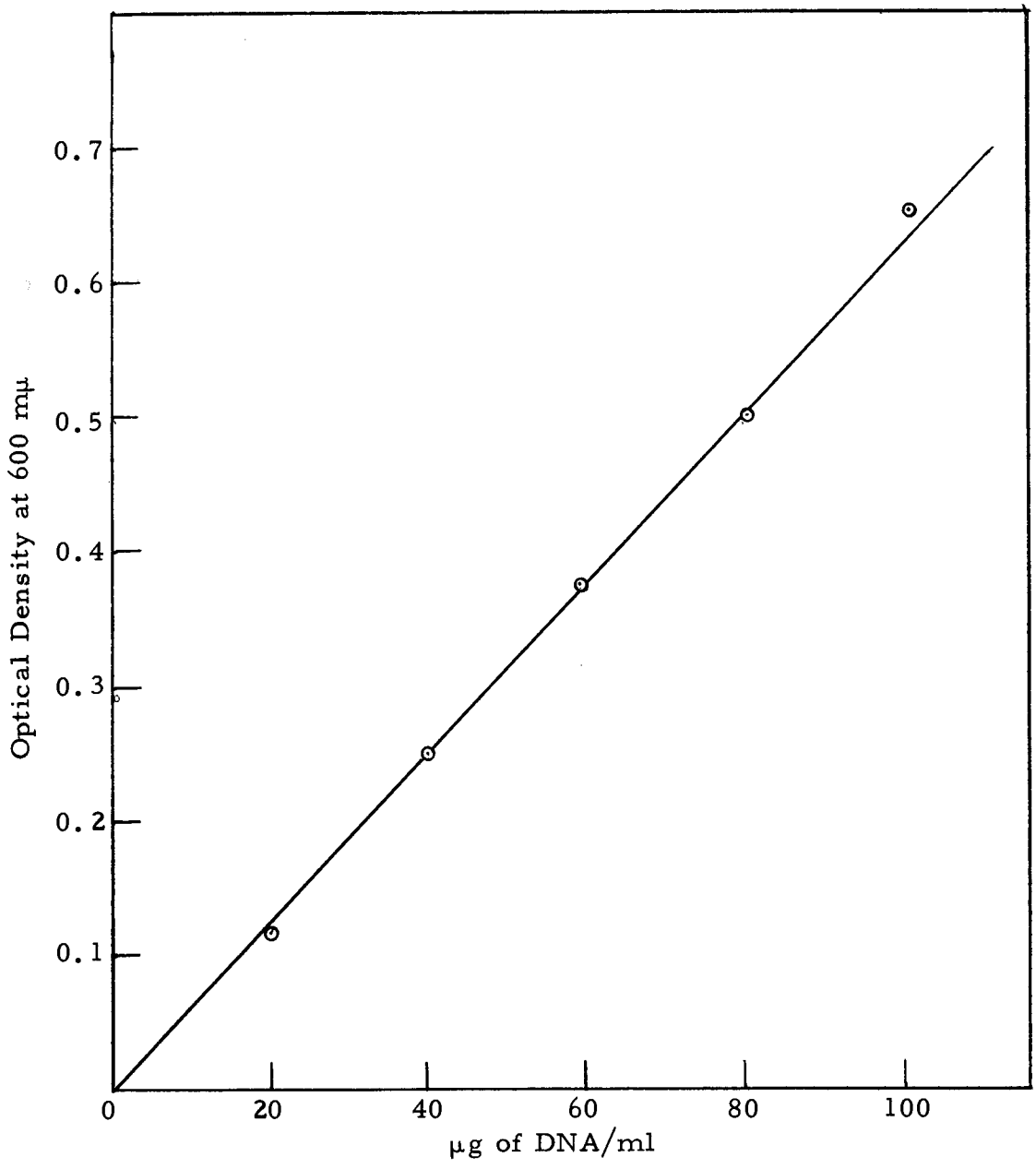

Figure 3. Standard reference curve for the determination of DNA concentration.

orcinol (Sigma Chemical Company) in 100 ml of concentrated HCl.

An aliquot of the sample was mixed with an equal volume of the orcinol reagent and heated at 100° C for 20 minutes. The tubes were cooled to room temperature, and the optical density at 670 mμ was read in a Zeiss spectrophotometer against a blank of standard saline citrate that had been treated in the same manner.

A standard curve (Figure 4) was prepared by dissolving purified yeast RNA (Sigma Chemical Company) in standard saline-citrate, pH 7, to give a concentration of 0.2 mg RNA per ml. Dilutions of this stock solution were made with standard saline-citrate to give concentrations of 10 to 100 μg RNA/ml. The orcinol reaction was carried out as described above, and the optical density at 670 mμ was plotted against the concentration of RNA.

Determination of Protein

The concentration of protein was determined by the folin reaction as described by Lowry et al. (1951).

The samples were diluted with sodium carbonate reagent (2% Na_2CO_3 in 0.1 N NaOH) so that the total volume in each tube was 0.5 ml. Two and a half milliliters of alkaline copper tartrate solution (1 part 1% $CuSO_4$, and 1 part 2% potassium tartrate in 100 parts of 2% Na_2CO_3 in 0.1 N NaOH) was added to each tube, and the tubes were mixed on a Vortex Jr. mixer. After standing at room

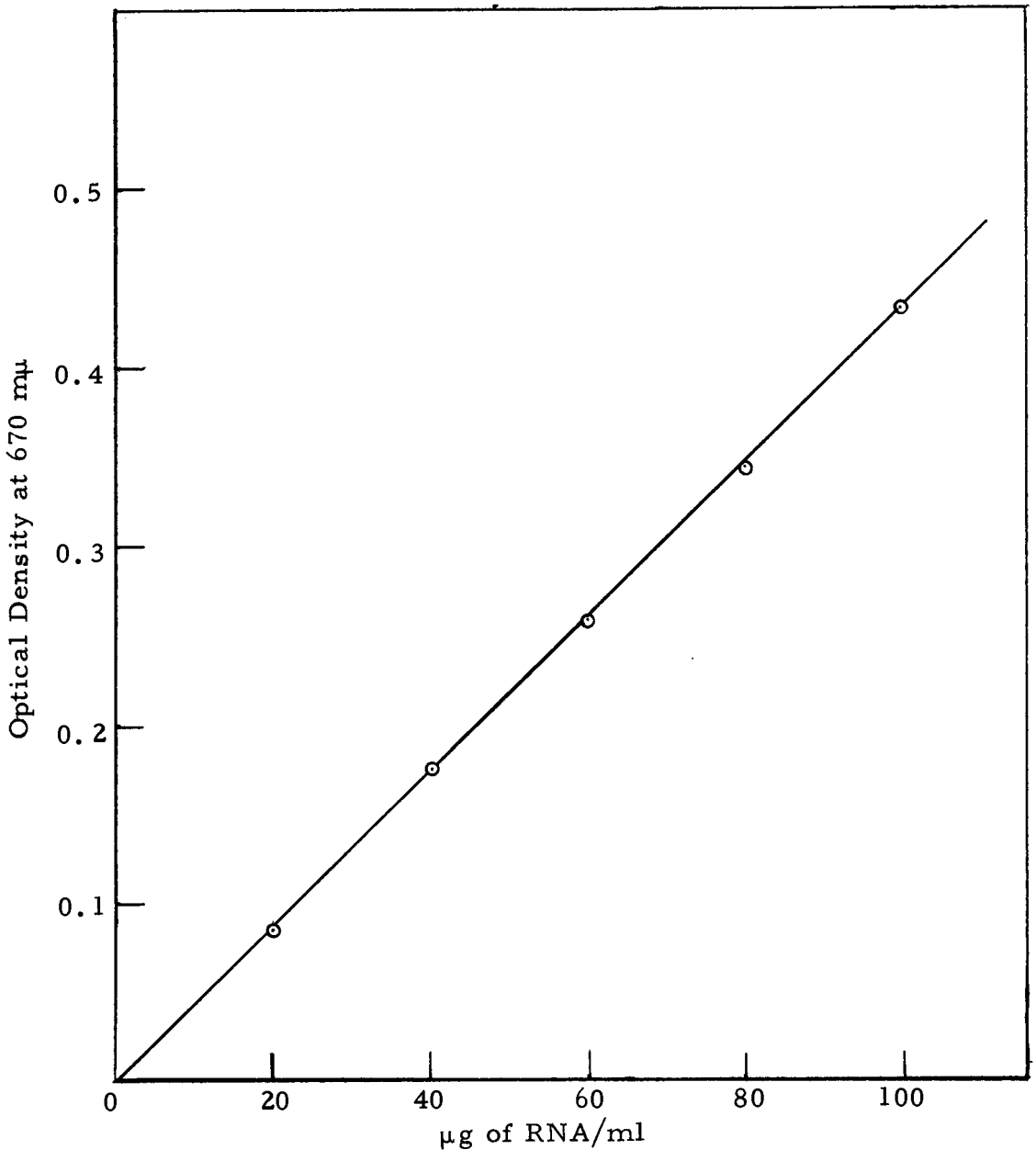

Figure 4. Standard reference curve for the determination of RNA concentration.

temperature for 10 minutes, 0.25 ml of folin reagent (concentrated folin diluted 1:1 with water; Fisher Scientific Company) was rapidly added to the tubes by holding the tubes against the Vortex Jr. mixer and pipetting at the same time. The tubes were allowed to stand at room temperature for 30 minutes after which time the optical density at 750 mμ was read in the Zeiss spectrophotometer against a blank of the sodium carbonate reagent that had been treated in the same manner.

A standard curve (Figure 5) was prepared by dissolving 40 mg of bovine serum albumin (Sigma Chemical Company) in 10 ml of 2% Na_2CO_3 in 0.1 N NaOH. This stock solution was diluted 1:26 with the sodium carbonate reagent, and dilutions were made using this solution to give protein concentrations between 8 and 80 μg/ml. The reaction was carried out as described above, and the optical density at 750 mμ was plotted against the concentration of protein.

Determination of Total Nitrogen

Total nitrogen was determined according to the procedure as described by Lang (1958). The digestion mixture was prepared by combining the following chemicals in the order given: K_2SO_4, 50 gm; selenium oxychloride, 2 ml; water, 250 ml; and concentrated H_2SO_4, 250 ml. Nessler's reagent was prepared by dissolving 68 gm of Nessler granules in 100 ml of water, followed by the

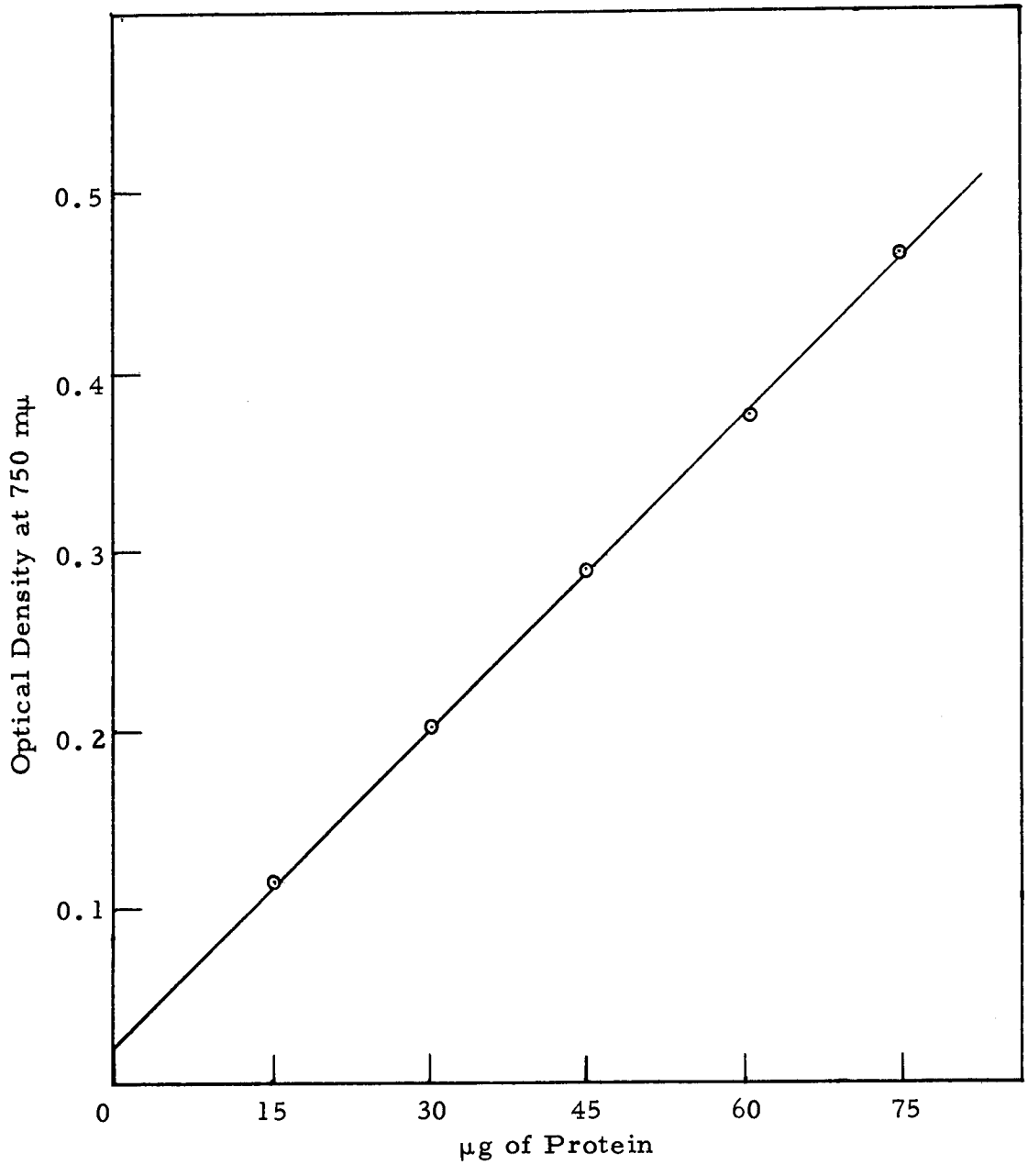

Figure 5. Standard reference curve for the determination of protein concentration.

addition of 850 ml of aqueous 10% NaOH, and of water to a final volume of 1000 ml. The reagent was allowed to stand, protected from the light, several days before it was used.

The samples were first digested by placing 0.5 ml of sample, 0.5 ml of water, and 0.2 ml of digestion mixture in clean 3 ml test tubes. The tubes were inserted into a digestion apparatus, consisting of an electric disk embedded into a sand bath, and digestion was carried out at 310 - 320° C for 1.5 hours or until the solutions became colorless. The contents of each tube were then made up to 10 ml with water, and an aliquot of 3 ml was transferred to another tube. The volume in these latter tubes was brought up to 4 ml with water, and 2 ml of Nessler's reagent was added to the tubes. The tubes were mixed on a Vortex Jr. mixer and allowed to stand at room temperature for 20 minutes. The optical density was read at both 420 mμ and at 500 mμ in the Zeiss spectrophotometer against a blank of distilled water that had been digested and treated in the same manner.

A standard curve (Figure 6) was prepared by dissolving 0.4716 gm of $(NH_4)_2SO_4$ in 100 ml of 0.2 N H_2SO_4 to give a final concentration of 1000 μg N/ml. Aliquots of this stock solution were diluted with water to give concentrations ranging from 10 to 50 μg N/ml. These standards were digested, and the reaction was carried out as described above. The optical density readings were plotted

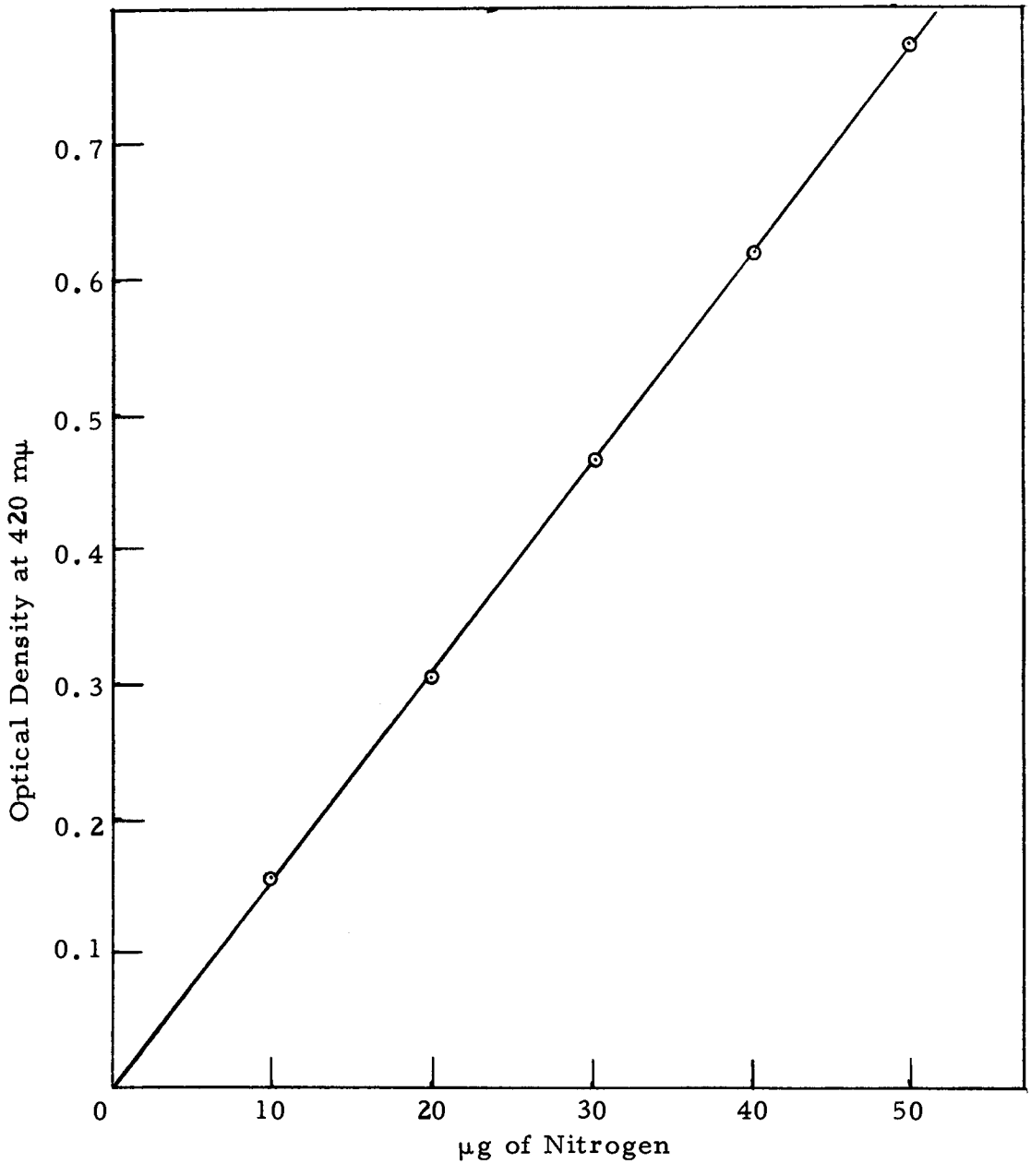

Figure 6. Standard reference curve for the determination of nitrogen concentration.

against the concentration of nitrogen.

Determination of Amino Acids with Ninhydrin

The amino acid content was determined by the ninhydrin reaction as described by Yemm and Cocking (1954). The ninhydrin reagent was prepared by diluting 5 ml of a 0.01 M potassium cyanide solution to 250 ml with methyl cellosolve. This solution was then mixed with a 5% (w/v) solution of ninhydrin (Nutritional Biochemical Corporation) in methyl cellosolve in the proportions of 1:5 respectively. The reagent was allowed to stand overnight before it was used.

The test was carried out by mixing 0.1 ml of sample with 0.1 ml of concentrated HCl and autoclaving the solution overnight at 121° C (15 lbs pressure). The volume was then made up to 5 ml with water. The samples were diluted with 0.2 M citrate buffer, pH 5, to give different concentrations of sample in a total volume of 1 ml. Five-tenths milliliter of the citrate buffer and 1.2 ml of the potassium cyanide-methyl cellosolve - ninhydrin reagent was added to each tube in the order stated. The tubes were well mixed, heated at 100° C for 15 minutes, and cooled to room temperature. Two milliliters of 60% ethanol was added to each tube, and the tubes were shaken. The optical density at 570 mμ was read in the Zeiss spectrophotometer against a blank of 0.5 ml of citrate buffer, pH 5,

that had been treated in the same manner.

A standard curve (Figure 7) was prepared by dissolving 2 mg of L-glutamic acid (Nutritional Biochemical Corporation) in 20 ml of distilled water. Dilutions of this stock solution were made with citrate buffer, pH 5, to give concentrations of 1 to 20 μg L-glutamic acid/ml. The reaction was carried out as described above, and the optical density at 570 mμ was plotted against the concentration of amino nitrogen.

Determination of Phosphorus

Total phosphorus and inorganic phosphorus determinations were carried out according to the procedure of Lowry et al. as modified by Young (personal communication).

Total Phosphorus

Determination of total phosphorus consisted of mixing together 0.1 ml of sample, 0.1 ml of water, and 0.2 ml of digestion mixture (30.6 ml of concentrated H_2SO_4 and 6.7 ml of 70% $HClO_4$ diluted to 100 ml with water) in a 3 ml test tube. The tubes were placed in a sterilizing oven and digested for 1 hour at 95° C, and then for 2 hours at 165° C. The tubes were cooled to room temperature and 2 ml of development reagent I (1 ml of M NaOAc, 1 ml of 2.5% NH_4 molybdate, 8 ml distilled water, and 100 mg of ascorbic acid) was

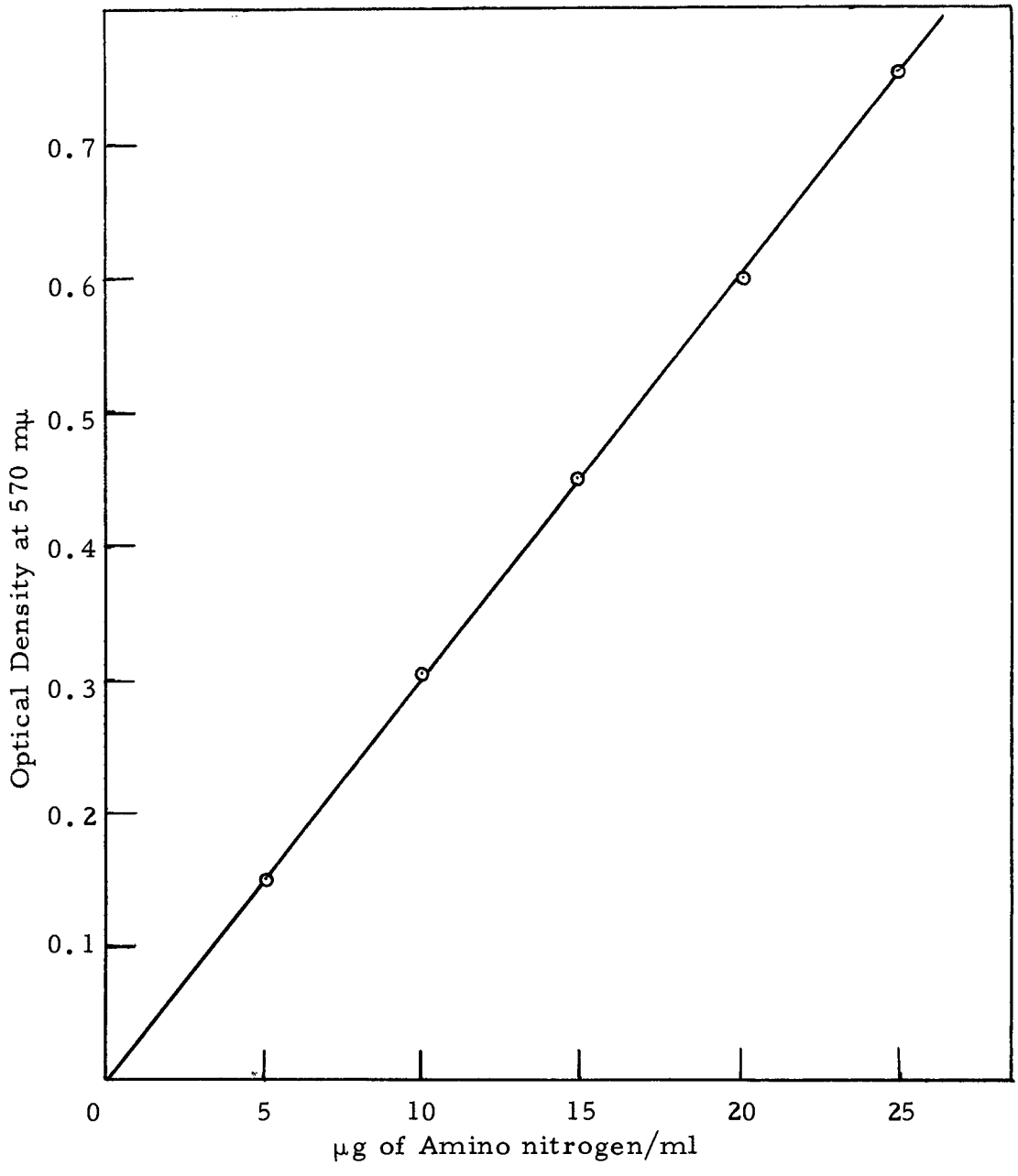

Figure 7. Standard reference curve for the determination of amino nitrogen concentration.

added to each tube. The tubes were incubated for 1.5 hours at 37° C, and the optical density at 820 mμ was read in the Zeiss spectrophotometer against a blank of distilled water that had been digested and treated in the same manner.

A standard curve (Figure 8) was prepared by dissolving 1.3613 gm of KH_2PO_4 in 1 liter of distilled water. This stock solution was diluted 1:100 and 1:50 in distilled water to give phosphorus concentrations of 100 mμmoles P/ml and 200 mμmoles P/ml respectively. Aliquots of these solutions were put into 3 ml test tubes to give a total volume of 0.2 ml containing 10, 20, and 40 mμmoles P/ml. Two-tenths milliliters of digestion mixture was added to each tube, and the reaction was carried out as described above. The optical density at 820 mμ was plotted against the concentration of phosphorus.

Inorganic Phosphorus

Determination of inorganic phosphorus was carried out by mixing 0.1 ml of sample, 0.1 ml of water, and 1.8 ml of development reagent II (0.7 ml of 10 N H_2SO_4, 0.6 ml of 2.5% NH_4 molybdate, 7.7 ml water, and 300 mg of ascorbic acid) in a 3 ml test tube. The tubes were incubated for 1.5 hours at 37° C, and the optical density at 820 mμ was read in the Zeiss spectrophotometer against a blank of 0.2 ml distilled water that had been treated in the same manner.

A standard curve (Figure 9) was prepared by diluting the stock

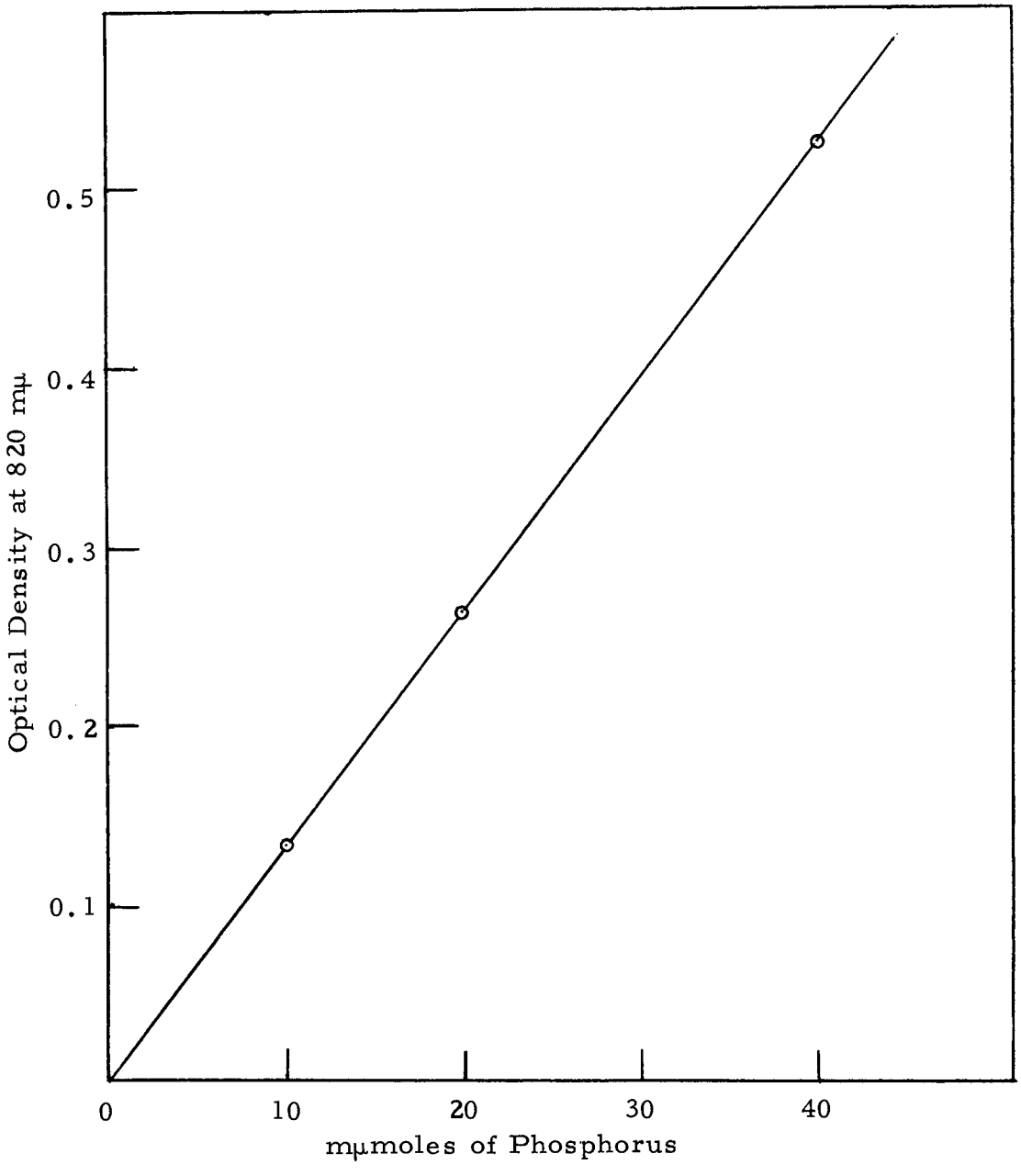

Figure 8. Standard reference curve for the determination of organic phosphorus concentration.

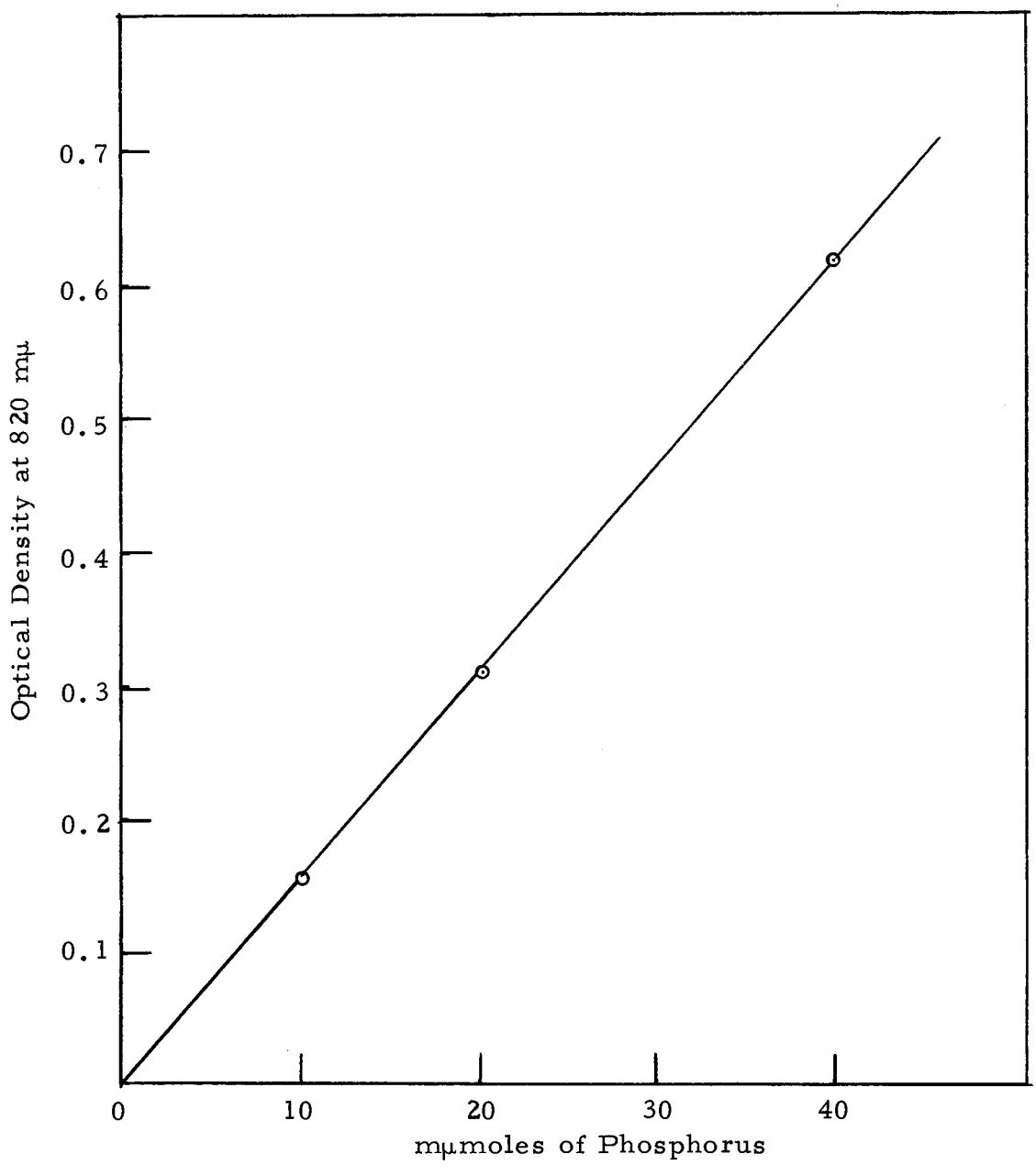

Figure 9. Standard reference curve for the determination of inorganic phosphorus concentration.

solution of KH_2PO_4 as described in the total phosphorus determination. Total volumes of 0.2 ml containing phosphorus concentrations of 10, 20, and 40 mμmoles P/ml were placed in 3 ml test tubes. The reaction was carried out as described above, and the optical density at 820 mμ was plotted against the concentration of phosphorus.

Amino Acid Analysis

Amino acid analyses were kindly carried out by Dr. R. R. Becker, Science Research Institute, Oregon State University, using a Beckman Spinco Model 120B amino acid analyser.

RESULTS AND DISCUSSION

Centrifugation of the mixture obtained after lysing the bacterial cells with sodium lauryl sulfate and shaking the lysate with chloroform-isoamyl alcohol results in a separation of the mixture into three layers: an aqueous top layer containing the nucleic acids, a gelatinous middle layer, and a clear bottom layer containing the solvent. The gelatinous middle layer consists of cellular proteins, carbohydrates, lipids, cell wall material, and some RNA and DNA. These middle layers were treated with 0.25 N HCl to separate the proteins into acidic and basic protein fractions, and these fractions were then hydrolyzed and analyzed by two-dimensional paper chromatography to identify the constituent amino acids.

Table 1 shows the proportions of acidic and basic material obtained upon fractionating the middle layers. The designation "first extract" refers to the gelatinous middle layer obtained after the first treatment of the cell mixture with chloroform-isoamyl alcohol, and the designation "combined extracts" refers to the gelatinous middle layers obtained from the second, third, and fourth treatments of the aqueous layer with chloroform-isoamyl alcohol. It can be seen from the table that, by weight, the basic fractions comprise most of the material of these middle layers. The amount of acidic material present in the extracts increases with an increase in the growth time

Table 1. The proportions of acidic and basic material obtained upon fractionating the middle layers with 0. 25 N HCl.

Culture	Extract	Acidic Fraction (gms)	Basic Fraction (gms)	Acidic Fraction % of Initial Total	Basic Fraction % of Initial Total	Ratio of Acidic Fraction/Basic Fraction
5 hours	First	0. 0959	0. 5657	14	83	0. 17
	Combined	0. 0132	0. 3386	3	83	0. 04
8 hour	First	0. 1403	0. 4859	19	65	0. 29
	Combined	0. 0081	0. 3241	2	80	0. 03
11 hour	First	0. 4207	0. 3910	43	40	1. 1
	Combined	0. 0411	0. 3348	9	76	0. 12

of the cultures. The increase of acidic material could be due to the formation of a poly-β-hydroxybutyric acid which B. subtilis stores. The formation of this compound is probably responsible for the high amount of acidic material present in the first extract of the 11 hour culture. The fact that the basic fractions all contain a high amount of material indicates that some compound or compounds in the cell mixture have properties intermediate between nucleic acids and protein, and during the extraction procedure these compounds are distributed between the two layers in equilibrium fashion. During each subsequent treatment, part of the compounds are brought down into the middle layer.

Table 2 shows the amino acids present in each fraction as identified by paper chromatography. In general, the same amino acids were present in all the fractions. It is interesting to note that while the basic fractions contained the most material by weight, very little of this material appeared to be protein in nature. Amino acids were very hard to identify in the basic fractions even when a larger volume of material was used for spotting on the chromatograms. The eight hour culture contained the highest amount of basic proteins. These observations suggest that the basic fractions consist mainly of salts and high molecular weight compounds other than proteins. Although it might be argued that the treatment of the middle layers with acid was not sufficient to extract the basic proteins, this seems

Table 2. Amino acids present in the acidic and basic protein fractions extracted from the middle layers.

Culture	Fraction	Amino Acids*																			Amount spotted in mgs.
		Cys	Asp	Glu	Lys	Arg	His	Ser	Gly	Thr	Ala	Tyr	Val	Met	Ileu	Leu	Try	Phe	OH-Pro	Pro	
5 hour First layer	acidic	(+)**	+++	++	+++	+++		+++	+++	++	+++	+	+		+	+		+			0.2
	basic	(+)	(+)	(+)	(+)	(+)		(+)	(+)	(+)	(+)		(+)		(+)	(+)					0.5
5 hour Combined layers	acidic										+		+		+	+					0.2
	basic		+	(+)	+	(+)		+	+		(+)										0.2
8 hour First layer	acidic	++	+++	+++	+++	+++	++	+++	++	++	++	+	+		+	+		+			0.2
	basic	+	++	++	++	++		++	+	+			+		+	+		+			0.2
8 hour Combined layers	acidic		+	++	+	+	+	++	++	(+)	+		+		(+)	+					0.2
	basic	+																			0.5
11 hour First layer	acidic	(+)	+++	+++	+++	+++		++	+	++	++	+	+		+	+		+			0.2
	basic		(+)	(+)		(+)		(+)	(+)		(+)				(+)	(+)					0.5
11 hour Combined layers	acidic	+	+++	+++	+++	+++	(+)	+++	++	++	++	+	+		+	+		(+)			0.2
	basic																				0.5

*The intensity of the color produced by each amino acid is designated as +, ++, or +++ in order of faintest to darkest color.

**Parentheses indicate that the amino acid spot was barely detectable.

unlikely since this method has been used successfully by other workers (Butler and Godson, 1963; Leaver and Cruft, 1966) to extract basic proteins from whole cells and from ribosomes. It has also been shown by Leaver and Cruft (1966) that basic proteins constitute only 0.32 to 0.96% of the total proteins of the bacterial cell.

One of the problems in studying deoxyribonucleoproteins is to be certain that the DNA-protein complex isolated is a true entity and not an artifact of the isolation procedure. With the methods so far used to study bacterial nucleoproteins, the existence of a definite DNA-protein complex has been hard to prove. It is assumed that if bacterial DNA were associated with a protein, there would be a firmer binding or attraction of this protein to the DNA than that of other proteins, and the associated protein would not be as easily removed from the DNA as would other cellular proteins, as long as dissociation conditions were kept to a minimum. During the extraction procedure, precautions were taken to prevent dissociation of any protein from the DNA complex by maintaining the salt concentration below 1 M. Based upon the Lowry and diphenylamine tests, the "DNA/protein" ratios of the three samples before dissociation of the DNA complexes were 1:0.33, 1:0.35, and 1:0.36 for the 5, 8, and 11 hour cultures respectively. The ultraviolet absorption spectra of these three samples are shown in Figures 10, 11, and 12.

The ultraviolet absorption spectra of the DNA obtained from

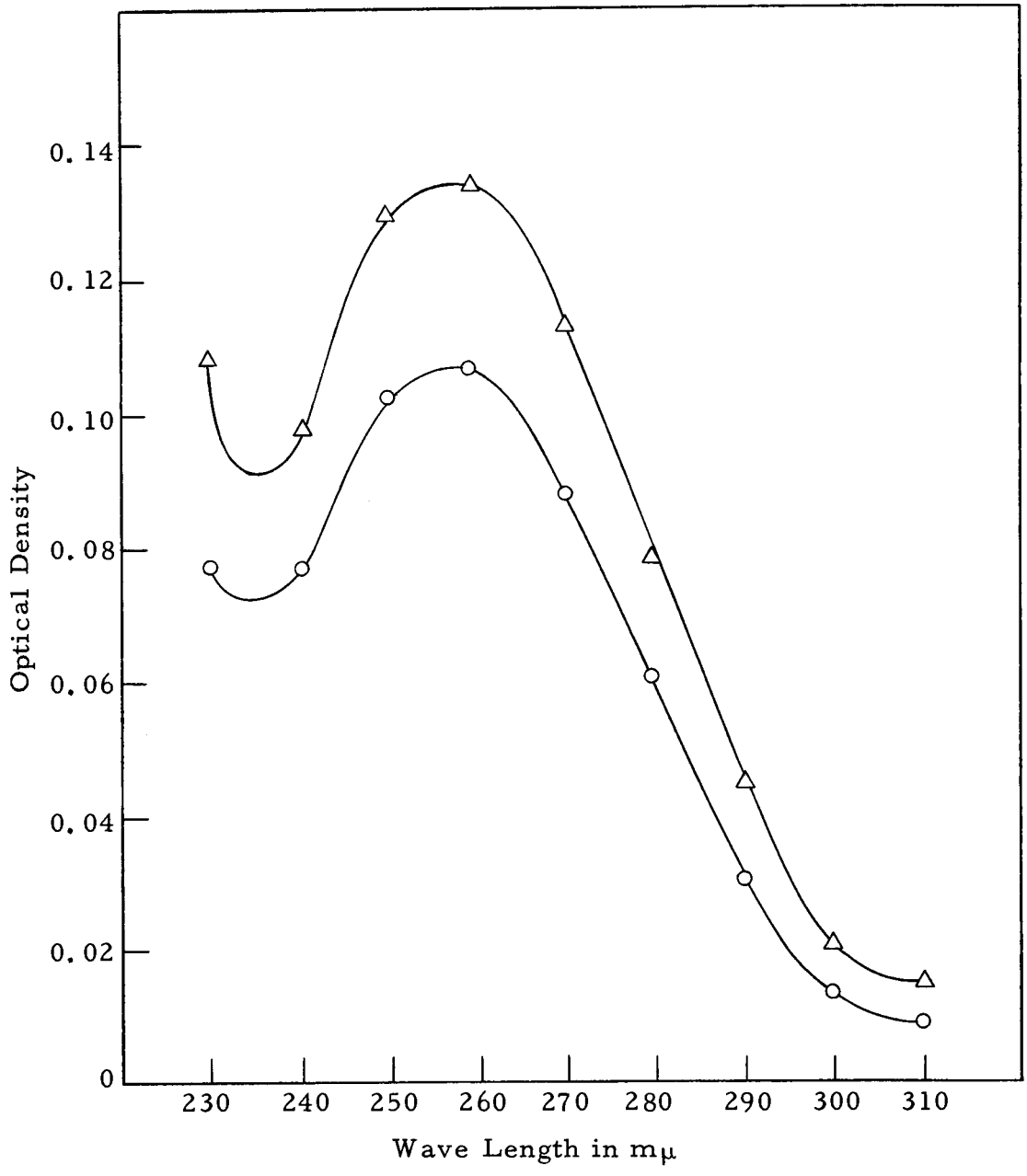

Figure 10. Absorption spectra of the DNA complex (o) and the dissociated DNA (Δ) isolated from the five hour culture.

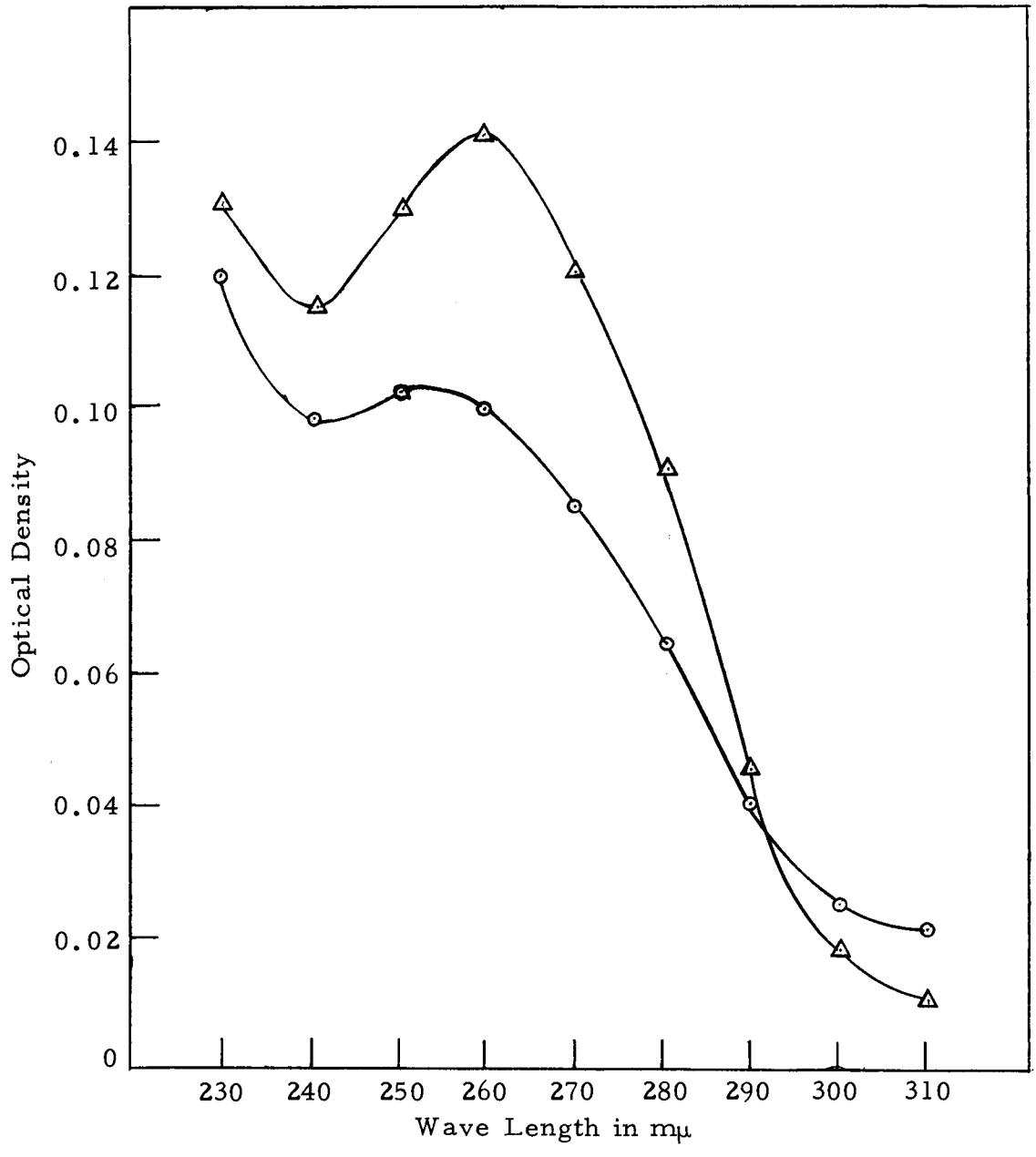

Figure 11. Absorption spectra of the DNA complex (○) and the dissociated DNA (△) isolated from the eight hour culture.

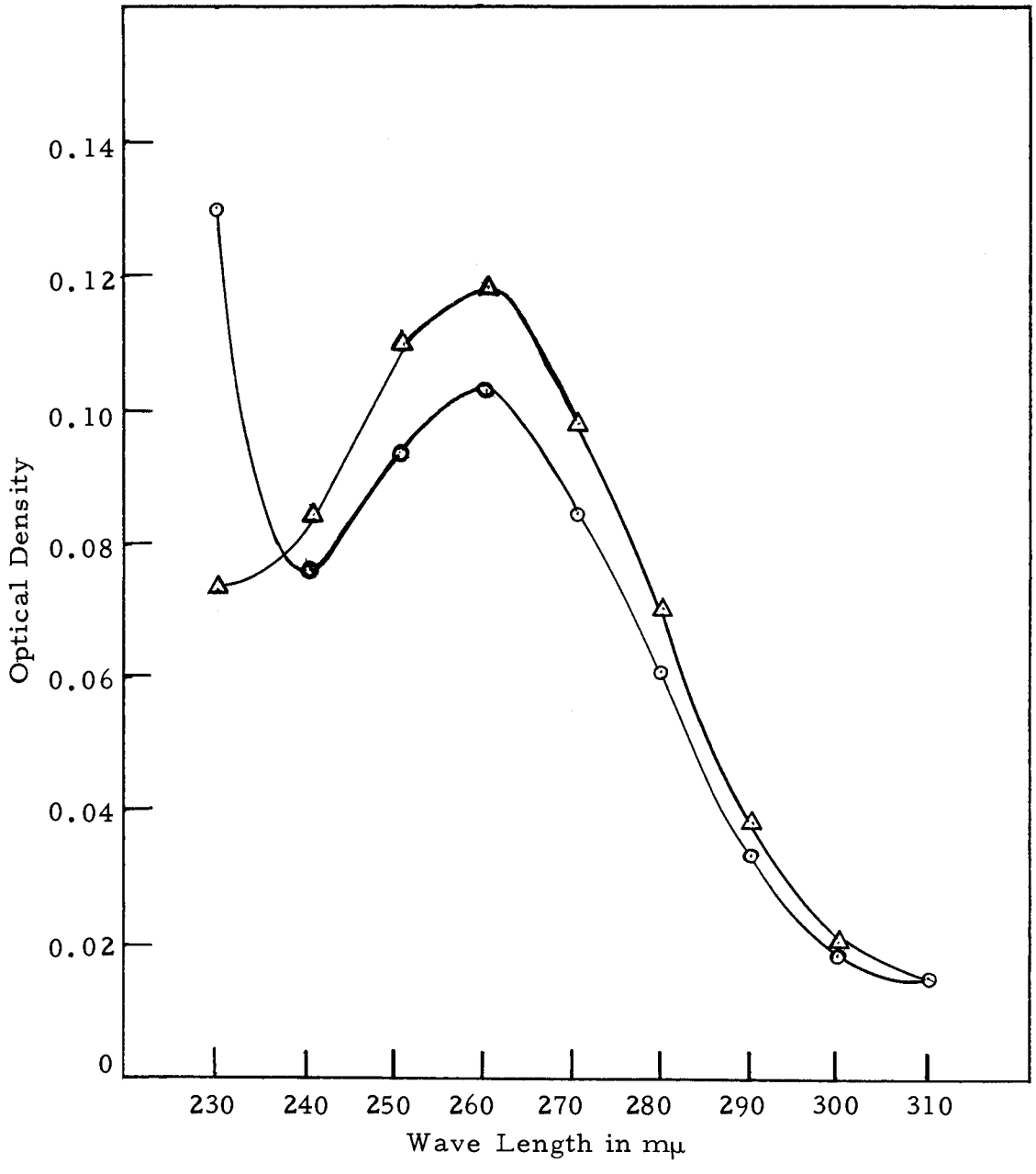

Figure 12. Absorption spectra of the DNA complex (o) and the dissociated DNA (Δ) isolated from the eleven hour culture.

the three samples after dissociation of the DNA complex in 3 M NaCl are shown in Figures 10, 11, and 12. The absorption spectra of the samples taken from the 8 hour culture and from the 11 hour culture before treatment with 3 M NaCl are not indicative of pure DNA. However, after treatment with 3 M NaCl, the curves represent those obtained with free DNA, suggesting that the salt treatment did remove some material that absorbed in the 230 to 240 mμ range. The salt treatment did not affect the absorption spectrum of the samples taken from the five hour culture. The 280 to 260 ratios of the three samples before and after treatment with 3 M NaCl are given in Table 3. The low ratios indicate that very little protein containing either tryptophan or tyrosine was present in either the DNA complexes, or in the dissociated DNA fractions of each of the samples. It has been noticed, however, that in bacterial lysates there is very little absorbing material present at 280 mμ as compared to that of higher organisms (Fraser, personal communication). For this reason, a 280 to 260 ratio may not be valid in studying bacterial preparations. The presence of absorbing material in the 230 to 240 mμ range suggests the presence of a protein containing no aromatic amino acids.

A Lowry test of the material dissociated from the DNA complexes showed less than 12 μg of "protein"/ml to be present in each of the samples. The basic fractions extracted from these

samples were analyzed for total nitrogen, organic phosphorus, and amino acids (Table 4). The ultraviolet absorption spectra of these basic fractions are shown in Figures 13, 14 and 15. All three samples were similar in chemical composition and in ultraviolet absorption properties. Attempts to identify amino acids in these samples by means of paper chromatography proved unsuccessful.

Table 3. The 280/260 ratios of the undissociated DNA complexes and of the DNA obtained after dissociation of the complexes with 3 M NaCl.

	5 hour culture	8 hour culture	11 hour culture
DNA complex	0.56	0.66	0.60
DNA	0.59	0.68	0.60

Table 4. Results of the chemical determinations performed on the basic fractions extracted from the material dissociated from the DNA complexes.

Culture	Total Nitrogen test (mg N/ml)	Ninhydrin Test (mg Amino N/ml)	Phosphorus Test (mg P/ml)
5 hour	11	20.2	0.0037
8 hour	10.2	19.8	0.0043
11 hour	11.2	21.6	0.0029

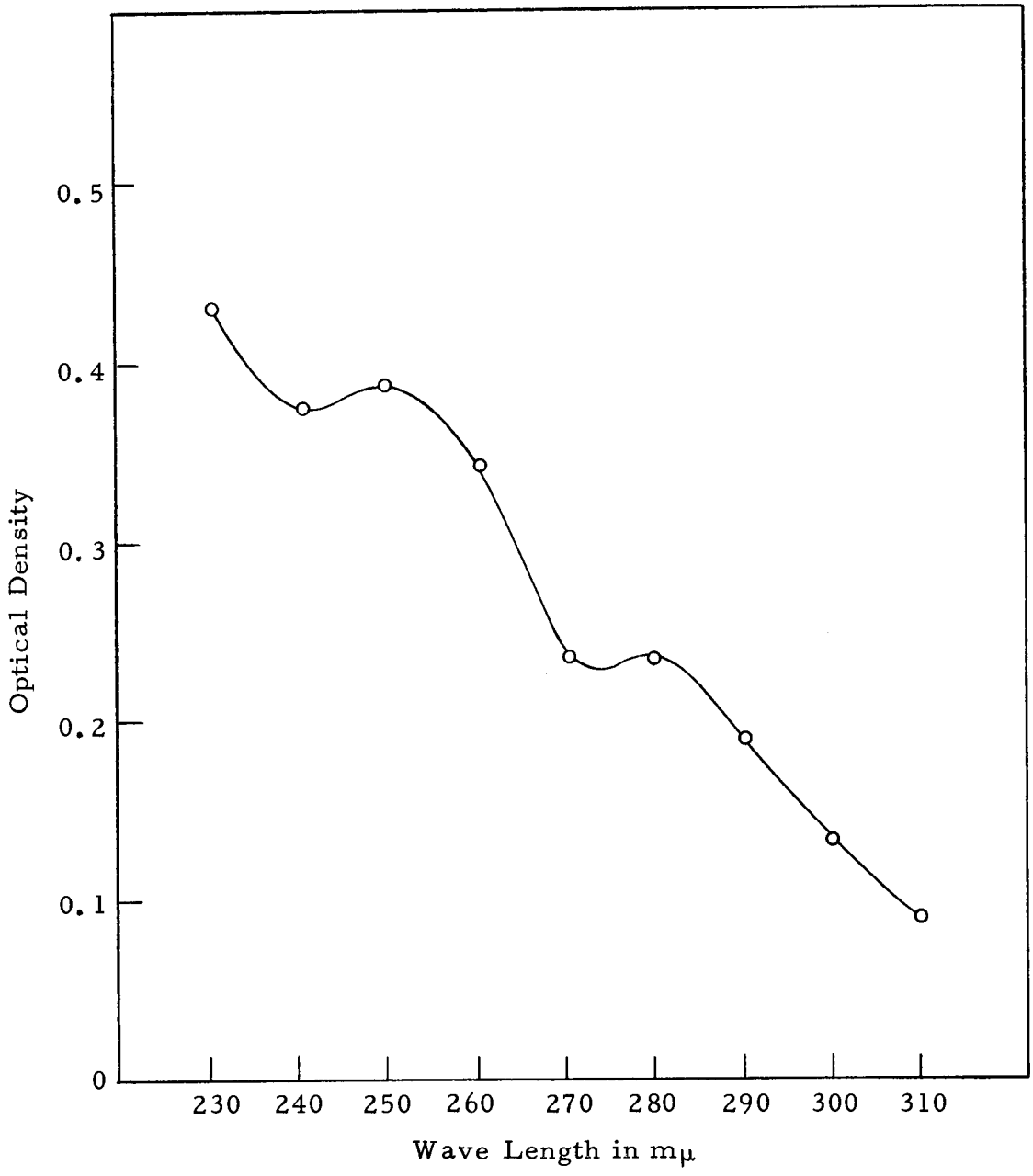

Figure 13. Absorption spectrum of the basic material dissociated from the DNA complex isolated from the five hour culture.

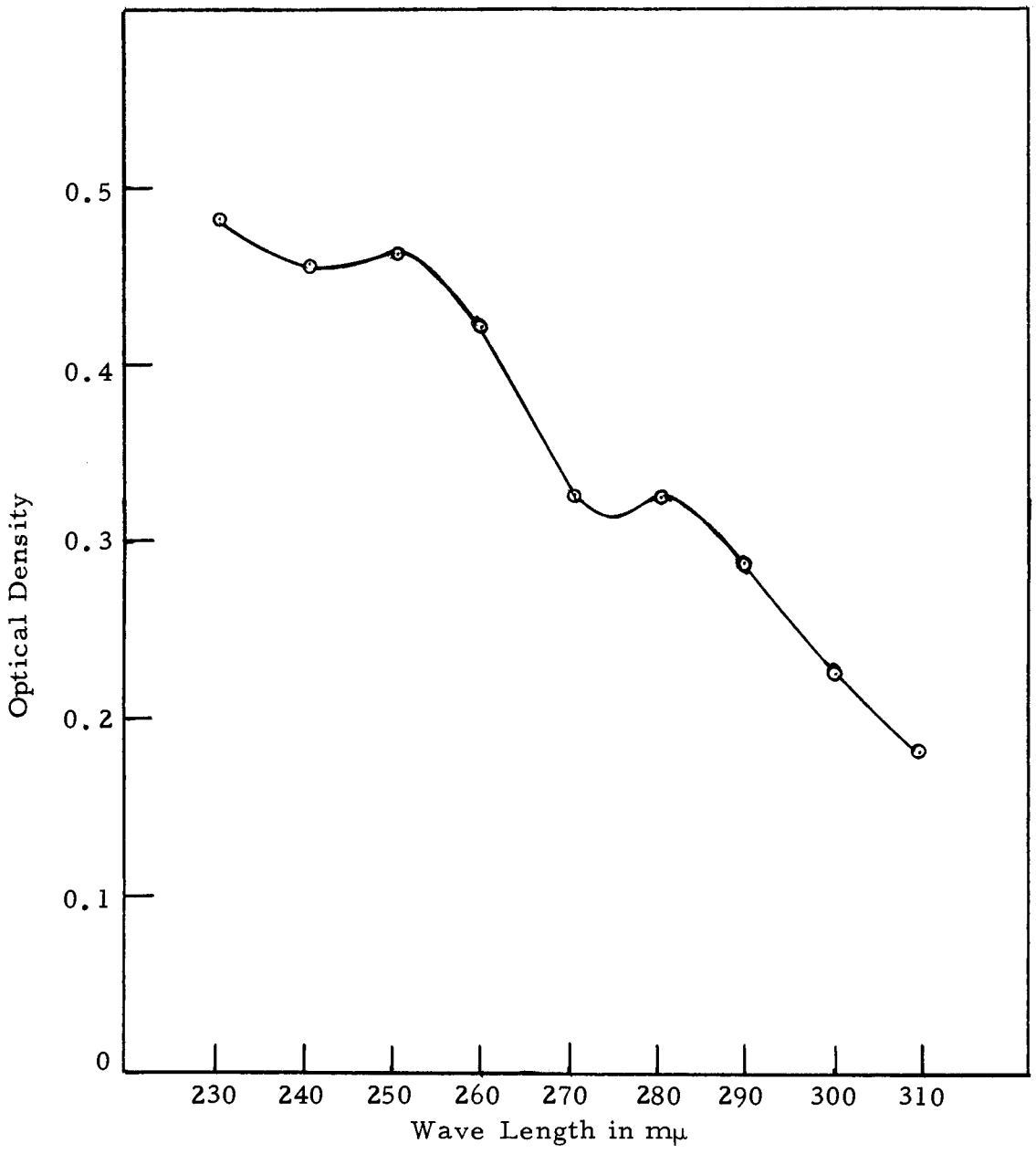

Figure 14. Absorption spectrum of the basic material dissociated from the DNA complex isolated from the eight hour culture.

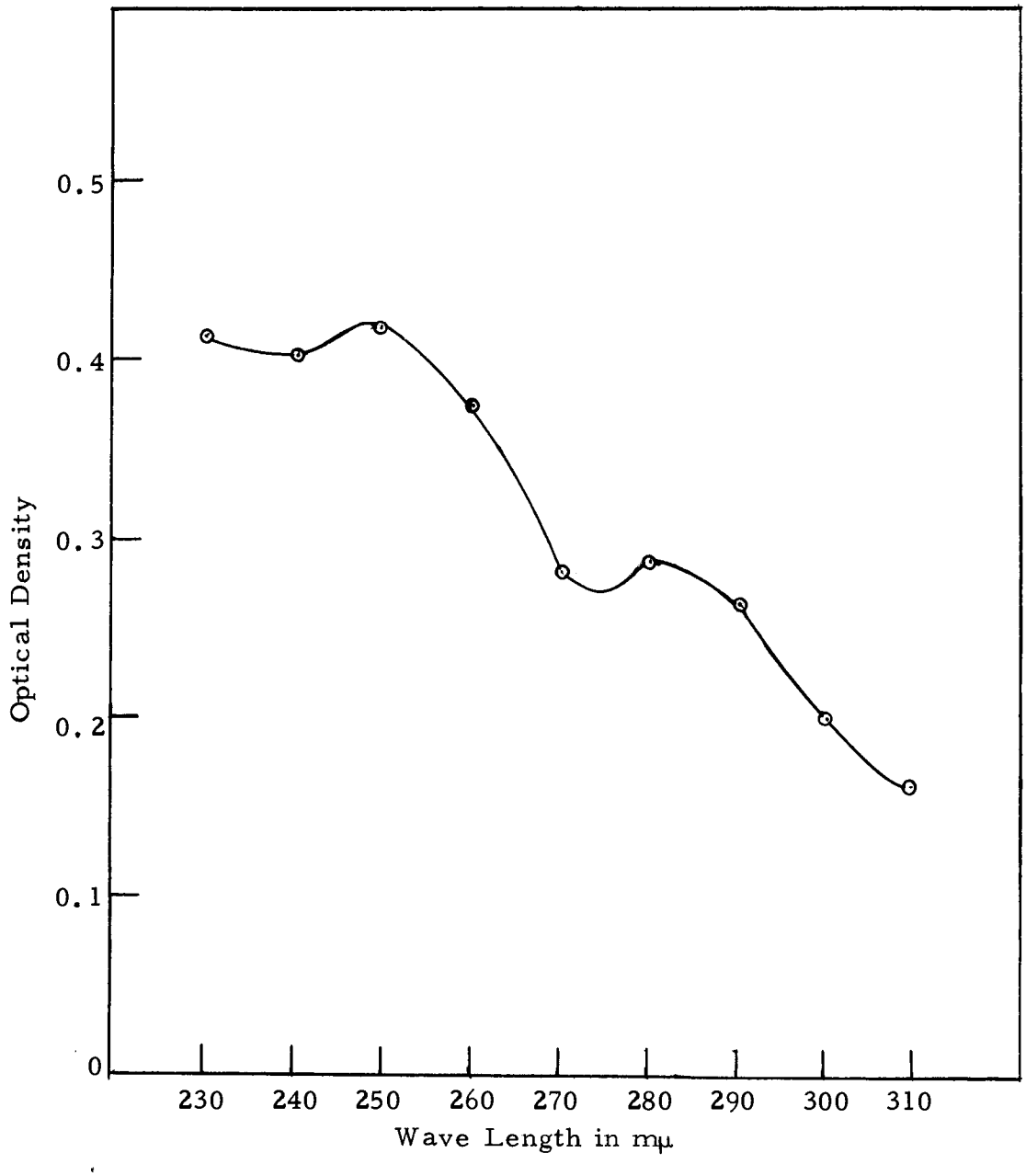

Figure 15. Absorption spectrum of the basic material dissociated from the DNA complex isolated from the eleven hour culture.

Amino acid analysis of the basic fraction obtained after dissociation of the DNA complex isolated from the five hour culture showed the presence of trace amounts of all the amino acids except lysine, histidine, arginine, and 1/2 cystine (Figure 16A). Since the amino acids were present in such low amounts, it was possible that their presence could be due to contamination. To test this possibility, a reagent control was analyzed for the presence of amino acids. The same amino acid spectrum was found in the control, although in smaller quantities (Figure 16B). On this basis, it would be difficult to say whether the amino acids present in the basic fraction were real or were due to contamination.

To determine what amino acids were present before the dissociation treatment, an amino acid analysis was carried out on the DNA complex isolated from the eight hour culture (Figure 17). The results of the amino acid analysis of the undissociated DNA complex are shown in Table 5. All of the amino acids can be detected with the exceptions of lysine, histidine, arginine, and 1/2 cystine. Glutamic acid, glycine, alanine, and methionine were the only amino acids present in any significant amounts. Glycine, however, is usually considered a degradation product of purine bases during hydrolysis. The absence of lysine, histidine, and arginine indicates that the protein, if one is associated with the DNA, is not a histone.

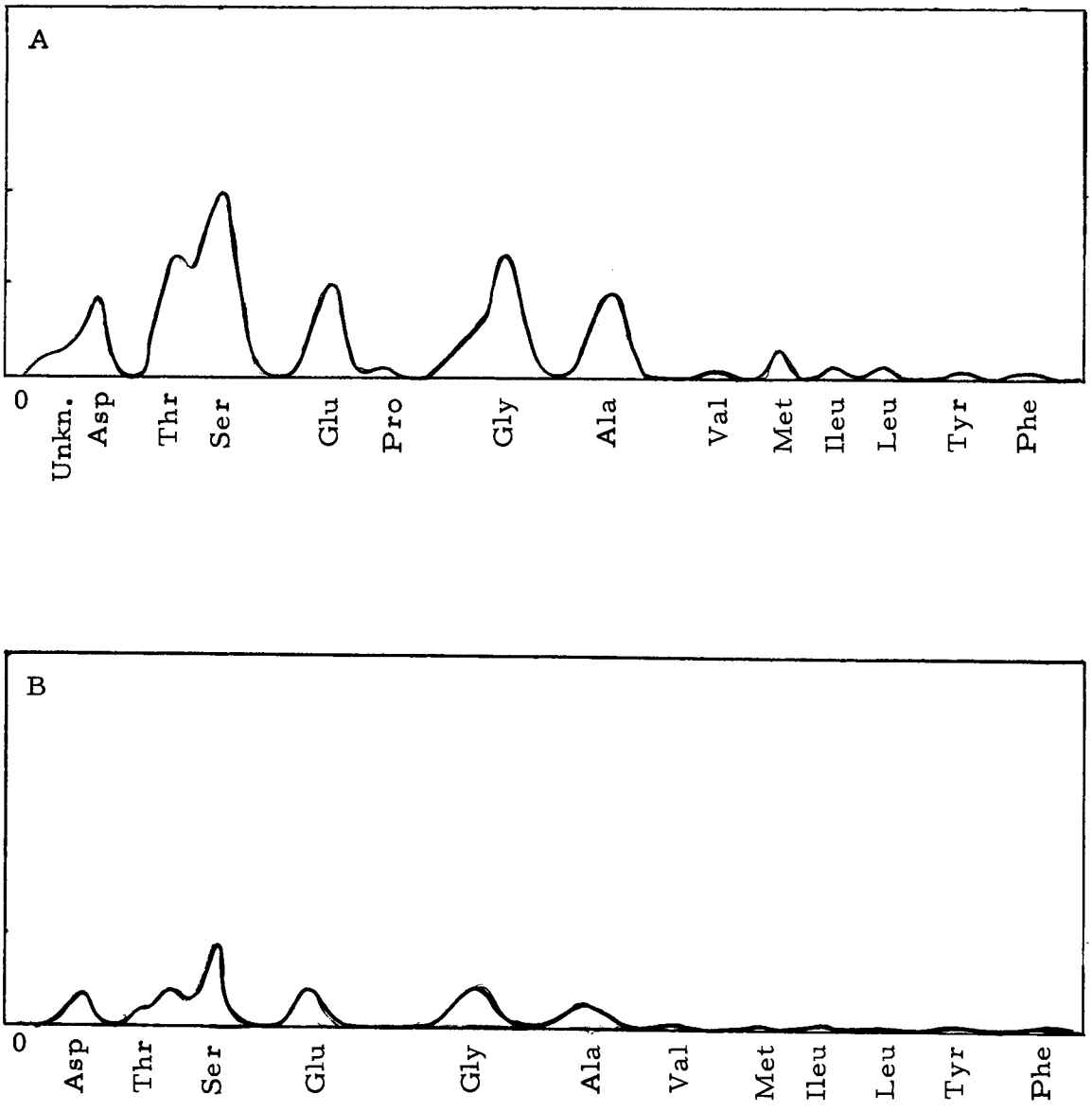

Figure 16. Amino acid analyses of (A) the basic material dissociated from the DNA complex isolated from the five hour culture and (B) of the reagent control.

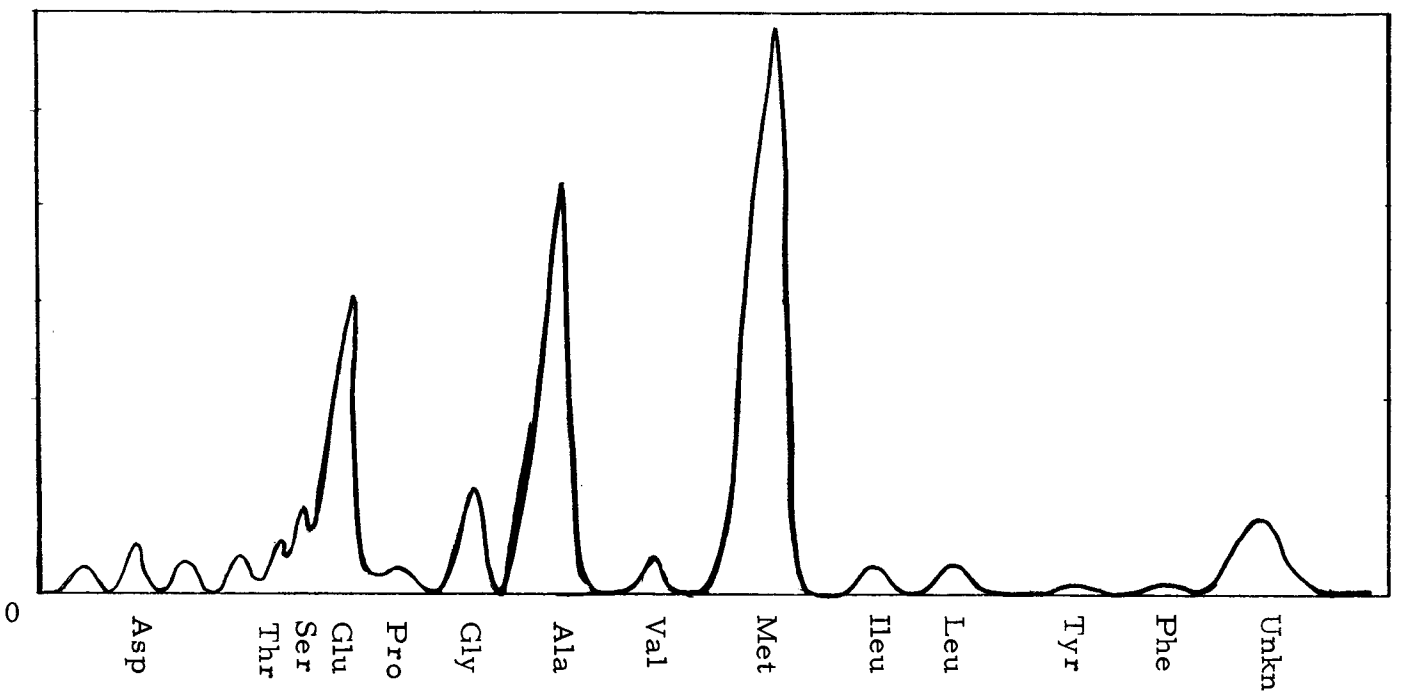

Figure 17. Amino acid analysis of the DNA complex isolated from the eight hour culture.

Table 5. Amino acid analysis of the undissociated DNA complex isolated from the eight hour culture

	μmoles
Nucleotides*	1.54
Lysine	None
Histidine	None
Arginine	None
Aspartic acid	Trace
Threonine	Trace
Serine	Trace
Glutamic acid	0.306
Proline	Trace
Glycine	0.108
Alanine	0.457
Half cystine	None
Valine	Trace
Methionine	0.381
Isoleucine	Trace
Leucine	Trace
Tyrosine	Trace
Phenylalanine	Trace

*540 μg DNA present, assume 350 to be average nucleotide molecular weight

$$\frac{540 \times 10^{-6} \text{ g}}{350 \text{ g/mole}} = 1.54 \text{ μmoles nucleotides}$$

It was found that a peak of NH_3 was present in the amino acid analysis of the basic protein fraction. The significance of this will be discussed later. An unknown peak was found in the amino acid analyses of both the undissociated DNA complex and in the basic fraction (Figures 16A and 17). The identity of this peak is unknown,

but it gives a pattern similar to that of glucosamine. However, glucosamine has an absorption maximum at 273 mμ whereas the absorption maximum of the basic fractions was around 280 mμ (Figures 13, 14, and 15).

It can be seen from the data that the samples taken from the three cultures were similar in both ultraviolet absorption properties and in chemical properties. This suggests that there was no difference in the type of material associated with the DNA at different times during the bacterial growth cycle. The data further suggests that a basic protein is not associated with the DNA under the conditions used.

The absence of protein in the DNA complex could be due to one of two factors. The protein was either dissociated from the DNA during the extraction procedure, or, alternatively, there was no protein associated with the DNA. Marmur's procedure for extracting DNA is quite severe, and it has been reported that treatment with $(NH_4)_2SO_4$, detergents, phenol, and organic solvents will precipitate proteins (Jones and Marsh, 1954; Bock and Gillchriest, 1958). However, it is not clear in the work cited whether this actually included nucleoproteins or removal of cellular proteins. There are also conflicting reports as to the effectiveness of these reagents in removing associated protein. The high amounts of glutamic acid, glycine, alanine, and methionine found in the undissociated DNA

complex are surprising, but it is very difficult to remove all traces of protein from the DNA. Glycine probably arises from the degradation of the purine bases of the DNA. The presence of the other amino acids is either an artifact of the extraction procedure, or they are in some way associated with the DNA. The individual amino acids could be linked through their amino groups to the phosphate groups of the DNA, or, alternatively, the amino acids could be present as peptides and the peptides joined to the DNA. The exact origin of these residual amino acids and whether this is a random or specific type of association is unknown. The absence of basic amino acids, even in trace amounts, indicates that a basic protein is not associated with the DNA, at least after isolation. Berns and Thomas (1965) found the amino acids glutamic acid, aspartic acid, glycine, alanine, threonine, and serine to be present in significant amounts in a DNA preparation extracted from H. influenzae using phenol.

Deoxyribonucleic acid from bacterial, viral, and mammalian sources, has not yet been isolated entirely free of amino acids. It is not clear whether the amino acid residues which are liberated from the DNA by acid hydrolysis are present as such in covalent linkage, or as covalently attached peptides, or as small proteins. These amino acid residues occur in small amounts (0.1 to a few percent), and their significance is not as yet completely understood (Bendich and Rosenkranz, 1963). The amino acid linkages are

believed to lend flexibility to the stiff main chain of the DNA, thus permitting the bending and folding of the DNA molecule. Polyamines have been found in combination with the DNA of the T-even phages. The function of these polyamines is thought to be to neutralize the acidic phosphate groups of the DNA and to help facilitate the tight folding of the DNA molecule to enable it to fit inside the phage head (Stent, 1963, p. 67-68).

Efforts to extract a basic protein from the DNA complexes were unsuccessful as shown by amino acid analyses. A Lowry test for protein indicated that only a very small amount of peptide or protein material had been dissociated from the DNA. Ionic strength alone may not be sufficient for removing protein from bacterial DNA since the amount of protein removed by this treatment seems to vary a great deal in different species (Zubay and Watson, 1959; Masui et al. 1962). Amino acid analysis showed that very little protein was present in the sample. If individual amino acids, or peptides, were associated with the DNA, these might have been removed by the 3 M NaCl. They would have then been dialyzed out during the subsequent dialysis steps. It is also possible that the amino acids were not removed by the NaCl treatment and were still attached to the DNA.

During the amino acid analysis it was found that a high peak of NH_3 was present in the basic fraction. The presence of NH_3

would account in part for the values obtained in the total nitrogen and in the ninhydrin tests. The ammonia could have resulted from the extraction procedure since NH_3 was used to adjust the pH of the samples. However, most, or all, of this NH_3 should have been removed during the subsequent dialysis and evaporation steps. Ammonia could have also been derived, in part, if the amino acids found in the samples were present as amides (eg. glutamine or asparagine), or if amines were present. Glucosamine, if present, would have broken down to form NH_3 under the conditions used.

As yet, basic proteins have not been found to be associated with bacterial DNA. The only report of a basic protein, similar to that of histones, associated with bacterial DNA is that of Bhagavan and Atchley (1965). However, it is possible that their basic-like protein came from ribosomal protein rather than from a DNA-protein complex. If this is true, it would also suggest that their DNP complex was an artifact and not a true entity. It is possible that the crude extract contained ribosomes which were not removed by the procedure used in their experiments. The DNP was then precipitated with 0.01 M $MgCl_2$. This concentration of $MgCl_2$ has been reported to cause aggregation and precipitation of ribosomes and ribonucleoproteins (Chao, 1957; Wang, 1961). Since ribosomes contain basic proteins (Waller and Harris, 1961; Leaver and Cruft, 1966), it is possible that their basic-like protein was derived from

ribosomal basic protein rather than from a DNA-protein complex.

Yoshikawa (1966), using B. subtilis, showed that a protein was associated with the DNA in slow growing cells with a generation time longer than three hours. This protein was not analyzed to determine if it was acidic or basic in nature. No protein was found to be associated with the DNA of either stationary cells or spores.

On the basis of the results reported here, and of those of other workers, it would seem that a nucleoprotein complex, found in cells of higher organisms, does not exist in bacterial cells. Chemical and physical studies (Wilkins and Zubay, 1959) indicate that a small amount of protein may be associated with the DNA of bacterial cells. Whether this protein is an artifact or a true entity is unknown. It is certain, however, that the protein is not a histone. If the sole function of histones is to act as gene regulators, then one might expect to find histones in bacteria. Failure to find histones in bacteria indicates that bacteria may have a different method of regulating gene expression. It may be that repression of bacterial genes is more temporary than that of genes in differentiated cells of higher organisms. However, as Yoshikawa suggests, a DNA-protein complex in bacteria may exist only during replication of the chromosome. This suggestion is plausible since some structural protein cell wall material seems to be involved in the regulation of bacterial chromosome replication (Lark, 1966).

SUMMARY

The purpose of this research was to determine if a basic protein was associated with the DNA of Bacillus subtilis W168, and to determine if there was a difference in the amount and type of protein associated with the DNA at different times during the growth cycle of the organism. The "DNA/protein" ratios of the DNA complexes isolated from cells grown for 5, 8, and 11 hours were 1:0.33, 1:0.35, and 1:0.36 respectively. Ultraviolet absorption spectra of the DNA complexes showed that, except for the five hour culture, some material was associated with the DNA. An amino acid analysis of the DNA complex isolated from the eight hour culture showed the amino acids glutamic acid, glycine, alanine, and methionine to be present in significant amounts. An unknown peak, possibly glucosamine, was also present.

The ultraviolet absorption spectra of the DNA samples obtained after dissociation of the DNA complexes in 3 M NaCl showed that some material had been removed from the DNA. This material, which contained very little protein, was separated into acidic and basic fractions. The three basic fractions were found to be similar when analyzed for total nitrogen, organic phosphorus, and amino acids. An amino acid analysis of the basic fraction obtained from the five hour culture showed the presence of trace amounts of all of the

amino acids except lysine, histidine, arginine, and 1/2 cystine. It can not be certain that their presence was not due to contamination. An unknown peak, corresponding to the peak found in the amino acid analysis of the DNA complex isolated from the eight hour culture, was also observed to be present in this analysis. In both cases, no basic amino acids were found to be present.

The cellular proteins, present at these three times, were studied for comparison with any protein found associated with the DNA. The middle layers resulting from the extraction procedure were separated into acidic and basic protein fractions, hydrolyzed, and analyzed for their constituent amino acids by two-dimensional paper chromatography. The amino acid spectrum was basically the same for all three cultures.

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