RIBONUCLEIC ACID METABOLISM AND BETA-GALACTOSIDASE INDUCTION IN NON-GROWING *ESCHERICHIA COLI*

By

John D. Loerch

AN ABSTRACT OF A THESIS

Submitted to
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The following investigations were carried out to study the effects of gratuitous induction on the metabolism of the ribonucleic acids of *Escherichia coli*. Incorporation of P^{32}-phosphate was used to examine the effects of beta-galactosidase induction by melibiose in non-growing cultures of the bacterium. The bacterial deoxyribonucleic acid, ribonucleic acid, and lipids were isolated from the ribosomal and soluble ultracentrifugal fractions obtained after sonic lysis of the bacteria, and the incorporation of radioactive phosphate into each component was determined. It was found that the inducer melibiose caused considerable stimulation in the synthesis or turnover of the ribonucleic acid and lipids, as measured by relative P^{32}-incorporation, but had no effect on the metabolism of deoxyribonucleic acid. All fractions examined were found to undergo a definite turnover even under non-growing conditions.

Alkaline hydrolysis of the ribonucleic acid fractions was followed by separation of the nucleotides which were then analyzed individually. These data indicated on the basis of radioactivity incorporation that the base ratios of

each of the ribonucleic acid fractions turning over under the influence of melibiose differed from the total base ratios of their parent fractions, but that the base ratios of these more active ribonucleic acid components were the same as those species of ribonucleic acid which persisted in turning over in the non-induced non-growing cultures. The implications of these findings with respect to the role of the inducer melibiose is discussed.

Chromatography of the ribonucleic acid obtained by phenol extraction of the ribosomal and soluble ribonucleic acid fractions on chitosan-cellulose columns showed the presence of specific sub-fractions of ribonucleic acid. In general, two major sub-fractions and two or three minor sub-fractions of ribonucleic acid were obtained from each of the ultracentrifugal fractions, and each exhibited by means of variations in P^{32}-incorporation a different metabolic response to the non-growing conditions and to the addition of inducer to the culture medium. One of the two major sub-fractions in each fraction of ribonucleic acid seemed to be influenced considerably more by the inducer than the other.

The effects of addition of the uridine analog 5-hydroxyuridine to the culture during induction were examined in the light of its previously-reported ability to inhibit the formation of beta-galactosidase. The observation was made that the analog was totally unable to inhibit synthesis of

the enzyme even in concentrations of one-hundred times that reported as being adequate for total inhibition of the enzyme's synthesis in growing cultures. If the assumption is made that the inhibitory action of the analog is dependent upon incorporation into newly-synthesized ribonucleic acid, it follows that the observed synthesis of beta-galactosidase in these cultures must be supported by a complete pre-formed enzyme-forming system which requires only the presence of the inducer for activation. There is apparently no requirement for an association of ribonucleic acid metabolism with the formation of the induced enzyme.

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VITA

The author was born March 21, 1932, in Chicago and received his secondary education at Redford High School, Detroit. He was graduated from Michigan State College in June of 1953 with a Bachelor of Science degree in Chemical Engineering.

After working for Dow Corning Corporation for one year, he was called to active duty as a Second Lieutenant in the United States Air Force in March of 1954. His service included one-half year as a student in radar electronics school, three years as an instructor in radar electronics and one year as Electronics Maintenance Officer on overseas duty in Japan.

Immediately following his release from active duty in June of 1958 he enrolled in the Graduate School of Michigan State University. In the course of his graduate training the author served for six months as a Special Graduate Research Assistant in Chemistry under an Atomic Energy Commission grant. This was followed by an appointment as a National Institutes of Health Predoctoral Fellow in January of 1960.

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INTRODUCTION

There are no boundaries on man's desire to exercise control over his environment. The results range in our time from actual attempts to reach the nearby planets on the one hand to attempts to understand the life processes of viruses on the other. The great gaps in knowledge concerning the control mechanisms which underlie the developmental processes of growth and maturation are gradually giving ground, and the associated phenomena of adaptation and control of biochemical pathways is under investigation in many laboratories. The picture that is gradually taking shape focuses attention on the enzyme spectrum within the cell as being the limiting factor for what the cell is and does. By altering this spectrum, it is likely that both the capabilities and functions of the cell could be varied within the limits inherently imposed by the genetic material. Thus it seems likely that the ability to control the development of an organism and the capacities of its constituent cells will be founded primarily on a complete knowledge of the biological mechanisms controlling the synthesis of enzymes.

There are many examples in the literature concerning the effects of environment on the enzyme spectra of various organisms. For example, Fitch and Chaikoff (1) have investigated the variations in activities of seventeen rat liver

enzymes caused by changing the animals from a non-hexose-containing to a glucose-containing diet. Their observation that a period of greater than three days was required for the enzyme activities to reach their new equilibrium levels indicates that changes in the rates of synthesis of the enzymes might be part of the explanation for the observed changes in activity levels. The mechanism by which these altered enzyme levels are brought about is obviously obscured by the use of multicellular experimental organisms.

The use of the inducible enzyme systems of micro-organisms offers many advantages in experiments designed to clarify the mechanism by which enzymes are synthesized. Besides the simplicity of the bacterial system compared to multicellular organisms and the great degree of control that may be exercised over the culture, synthesis of a specific enzyme may be initiated, interfered with and terminated at will during such experiments, and in certain cases the data obtained may be correlated with a vast body of information accumulated by other workers. Induced enzyme synthesis, or as it is sometimes called, "enzymatic adaptation," may be defined as an increase in the rate of synthesis of a particular enzyme relative to the rate of synthesis of total cell protein. This disproportionate increase is caused by exposing the cells to a compound (inducer) that is generally related structurally to the substrates of the given enzyme(2).

It shouldbe mentioned that the substrate and inducer functions of a given compound are quite distinct. Hogness

(3) has demonstrated that one compound may serve as a substrate for an inducible enzyme but be incapable of inducing it, whereas another may not be a substrate yet may be a very effective inducer. The latter instance is generally referred to as gratuitous induction, and the compound is called a gratuitous inducer. Strict use of this term implies that the only function of the compound is to cause the induction of the specific enzyme concerned. Generally the criterion of lack of culture growth in the absence of a potential carbon or energy source other than the inducer is applied in ascertaining the gratuity of a particular inducer.

In the past, several possible mechanisms have been proposed for the induction phenomenon. According to one hypothesis (4), the inducer supplies in some way a necessary unspecified ingredient for the completion of an active enzyme-forming system, and those enzymes which are normally synthesized at a rapid rate are assumed to have a corresponding endogenous inducer which is elaborated within the cell. The inducible enzymes are assumed to be those for which no endogenous inducer is present and which must therefore be supplied with an external inducer before rapid synthesis of the enzyme can occur.

The alternative possibility is that the enzyme-forming system for each inducible enzyme is complete even in the non-induced organisms, but that these systems are in a repressed state. Until recently this possibility was lightly regarded. In light of the specific discovery of Vogel (5)

of the phenomenon of enzyme repression, however, the hypothesis has attracted considerable attention (6). A classical series of experiments by Pardee, Jacob and Monod has recently culminated in a publication (7) which very strongly indicates on genetic grounds that induction is actually the release of repression. The role of the inducer in these systems appears to be that of an antagonist for the repressor, which is characterized as a specific cytoplasmic "messenger" (8). It is apparent from their experiments that a specific cistron is present in the genetic material of Escherichia coli which controls the bacterium's capability of synthesizing beta-galactosidase, and a related but different cistron controls the ability to elaborate the repressor.

Since it appears at the present time that there is one general mechanism by which all proteins are synthesized, it is of value to consider the present state of knowledge concerning these mechanisms. Presently accepted views place emphasis on the ribonucleic acids as being the means by which the genetic codes are translated into the synthesis of enzymes. It has recently been demonstrated by Roberts, et al. (9), that the protein of bacterial ribosomes becomes labeled with radioactive sulfate sooner than the soluble bacterial protein, and that with the passage of time this labeled ribosomal protein becomes transferred to the soluble fraction. The data duplicate what has been known to occur in mammalian ribosomes for some time (10, 11), and lead to the same implication of ribosomal ribonucleic acid at the site

of enzyme synthesis. This ribonucleic acid species is often spoken of as template ribonucleic acid.

A second species of ribonucleic acid, termed soluble ribonucleic acid because of its non-sedimenting behavior in the ultracentrifuge, has been shown to bind amino acids (12, 13, 14) in a step prior to their being incorporated into newly-synthesized protein.

Yet a third type of ribonucleic acid supposedly important in the synthesis of proteins has been recently described by Volkin (15). This deoxyribonucleic acid-like ribonucleic acid has been termed messenger ribonucleic acid because of its supposed function in the direct transfer of coded information from the gene to the template ribonucleic acid to be used in the synthesis of specific enzymes.

Besides these partially assumed and partially demonstrated roles of various species of ribonucleic acid in the synthesis of proteins, there is a considerable body of evidence which links ribonucleic acid metabolism with induced enzyme synthesis in particular. It has been shown in several instances (16, 17) that ribonuclease interferes with induced enzyme formation whether added prior to or during the induction period. On the other hand, addition of pre-formed ribonucleic acid isolated from induced cells to non-induced cultures causes a sudden synthesis of the inducible enzyme, with the rate of formation declining quite rapidly back to the original low level. The potency of the ribonucleic acid extract was found to be destroyed by ribonuclease.

In several experiments by Chantrenne (18, 19, 20) it was found by use of radioactive precursors of ribonucleic acid that increased incorporation of these compounds accompanies the induction process. The fraction of ribonucleic acid into which this increased incorporation occurred was not ascertained.

Numerous experiments have been performed similar to the ones by Pardee (21) and Monod, et al. (22), in which it was found that certain strains of bacteria which require purines, pyrimidines or inorganic phosphate for growth also require these compounds for induction. Later experiments indicated however that in some specific instances induced enzyme synthesis can take place even during starvation for nucleic acid precursors.

Further evidence for the immediate involvement of ribonucleic acid metabolism with the formation of induced enzymes comes from the work of Spiegelman, et al. (23). These workers report that the uridine analog, 5-hydroxyuridine, when introduced into growing cultures of Escherichia coli, completely inhibits the induced synthesis of beta-galactosidase. Similar findings for other inducible systems involving other micro-organisms have been reported by Creaser using 8-azaguanine and 2,6-diaminopurine (24, 25). It is presumed that these analogs of ribonucleic acid precursors are incorporated into sensitive ribonucleic acid fractions, thereby rendering these molecules incapable of performing their normal functions in the synthesis of the enzyme.

Although the evidence for the function of some portion of the total cellular ribonucleic acid in induced enzyme formation is convincing, no evidence has yet been presented as to the localization of the effects of induction on the metabolism of specific sub-fractions of the total cellular ribonucleic acid. Also the preceding effects had generally been observed in rapidly growing cultures, where secondary metabolic responses to shifts in protein and ribonucleic acid metabolism might be expected to obscure or even completely conceal the primary effects of the induction process. To circumvent the latter objection, some workers have attempted to approximate non-growing condition in their cultures. For example, Chantrenne (20) has used actively fermenting but non-dividing yeast cells for incorporation studies involving labeled ribonucleic acid precursors. However, his system required that the yeast be changed from anaerobic to aerobic conditions as a condition for induction, and it seems possible that the effects he observed are more related to this drastic change to aerobic conditions than to the induction process itself.

The discovery by Rickenberg and Lester (26) of the phenomenon of "preferential enzyme synthesis" provides another example of induction in non-growing organisms. They found in their experiments that the beta-galactosidase synthesizing system of Escherichia coli grown on a defined salts-glucose medium in the presence of a beta-galactosidase inducer

remained repressed until the medium was exhausted of its glucose. An explosive synthesis of the enzyme was seen to occur just at the time that exhaustion of available glucose had caused complete cessation of bacterial growth. If the inducer used was also a substrate of the enzyme, exponential growth soon resumed following the diauxic growth plateau. But when a gratuitous inducer such as melibiose was used, growth was unable to resume at the expense of the inducer even though beta-galactosidase synthesis proceeded vigorously. Measurements of total protein in these experiments showed that no measurable net synthesis of protein occurred once the medium glucose was exhausted. The net result was that in the presence of melibiose the rate of synthesis of beta-galactosidase was disproportionately increased relative to the rate of synthesis of total cellular protein.

These results make available a minimal system for the study of induced enzyme synthesis. A maximum of extraneous variables are eliminated because of the use of truly non-growing cultures and no outside stimulus is required to initiate the experimental conditions other than the addition of melibiose. Further advantages in the use of this system are found in the great body of experimental evidence and experience that has been collected concerning Escherichia coli in general and the enzyme beta-galactosidase in particular, thus eliminating much of the exploratory work that might have to be performed with a different system. The advantages of being to analyze the results obtained from an experiment

against the background of such a body of previous observations are also obvious.

The goal of the experiments to be described in this thesis was to examine by means of P^{32}-phosphate incorporation the metabolism of the ribonucleic acids during induced enzyme synthesis. The non-growing melibiose-induced system of Rickenberg and Lester was selected to be used in these studies to eliminate insofar as possible the effects of general cellular growth on ribonucleic acid synthesis and turnover.

In order to ascertain the effects of induction on specific sub-fractions of the cellular nucleic acids, the ribosomal and soluble fractions were isolated and analysed separately. In addition, the effects of 5-hydroxyuridine on the synthesis of beta-galactosidase were examined in this system. It was anticipated that the presence of this uridine analog might cause an alteration in the metabolism of the cellular ribonucleic acid which would be detectable under the conditions of the experiment, thereby possibly affording some clues as to the mechanism by which the compound inhibits the synthesis of beta-galactosidase.

It was also decided to examine the effects of the inducer on incorporation of P^{32} into the deoxyribonucleic acids and the phospholipids of the bacterial cells so that a comparison could be made among several of their constituent molecular species.

EXPERIMENTAL AND RESULTS

EXPERIMENTAL AND RESULTS

Maintenance and Culturing Requirements for Escherichia coli

Two viable slants of the bacterium Escherichia coli strain ML 30 were kindly provided by Dr. H. V. Rickenberg, University of Washington, for use in these experiments. In his words, "Strain ML 30 cannot utilize melibiose as a source of carbon and yet is effectively induced by it to produce beta-galactosidase. Strain ML 30 hails originally from Monod's laboratory at the Institut Pasteur." The bacteria were initially maintained and cultured on the defined salts-glucose medium of Monod, et al. (27), and the procedures used are described in Appendix I.

Difficulties arose in experiments involving the incorporation of P^{32}-phosphate into cellular components under non-growing conditions because of excessive dilution of the radioactivity by the phosphate in the medium. Increasing the amount of labeled phosphate used in the experiments appeared undesirable because of the relatively high levels of activity already being handled (1 mc. per liter). The alternative, reduction of the over-all phosphate concentration of the medium, was therefore investigated.

Initial attempts to lower the phosphate concentration while maintaining a constant ionic strength through the use of potassium chloride failed, probably because of insufficient buffering capacity of the new media. The low-phosphate, Tris (tris-hydroxymethyl amino methane)-buffered medium of Volkin and Astrachan (28) was substituted for the high-phosphate medium of Monod, et al., and was found to be entirely satisfactory for all of these experiments. This medium was thereafter utilized in the slant as well as for the inoculating and experimental media (see Appendix I).

One further difficulty arose in the early experiments. A very pronounced haze or white flocculent precipitate often developed in making up the culture medium. This haze interfered with the turbidimetric assay of culture growth to be later described. The source of the haze was traced to the use of Fischer reagent grade potassium phosphate (monobasic) and was thereafter eliminated by the use of the Baker brand of salt in its place.

Several experiments also were performed to determine the optimum amount of glucose to be used in the medium. The concentration finally selected gave maximum growth of bacteria without causing any side effects such as a salt deficiency to appear. This optimum amount was estimated by observing the family of growth curves which resulted as the initial glucose concentration of the medium was increased. The growth curves were found to develop anomalies when the glucose concentration was increased beyond this optimum level.

Estimation of Growth and Extent of Induction

The turbidimetric method of estimating the level of growth achieved by any particular E. coli culture is by far the easiest to apply of those employed and was used routinely in the following experiments. Its use allowed not only evaluation of past growth but also accurate predictions to be made of future growth. It allowed dilution techniques to be employed such that the exact volume of inoculum could be calculated which would be required to produce a desired bacterial concentration in an experimental culture at any particular time. This was made possible by the determination that the turbidity was directly proportional to the concentration in these cultures. This allowed precise planning of the experiments which in many cases required careful attention continuously throughout a twenty-four to thirty hour period, and allowed the ready use of equipment which was available only during certain hours of the day.

In the following experiments the turbidity of a culture was taken as the reading given by the culture on a Klett-Summerson photoelectric colorimeter using the 420 mu (blue) filter. The finding that the sensitivity of the measurement increased as the wavelength of the light was decreased led to the selection of the blue filter as the standard. In a particular medium and at a fixed temperature a plot of the Klett units measured versus the length of time

the culture had been growing gave a typical and highly reproducible growth curve for the bacteria.

The remaining two criteria of growth were based on determinations of the total protein and the total ribonucleic acid present in aliquots removed from the growing culture. These criteria were employed to furnish information concerning the quantities of total bacterial protein and ribonucleic acid to be found in the culture at any particular time, thus allowing a comparison to be made of these results with the turbidimetric method of estimating growth. Even more important, however, it made possible the evaluation of the overall effects of the inducer on these cellular components in the non-growing cultures used in the experiments which follow. Figure 1 on page 16 demonstrates the appearance of these growth curves both for induced and non-induced cultures.

Before reproducible assays for protein, ribonucleic acid and beta-galactosidase could be run on the cultures, it was found necessary to lyse the bacteria. The procedure of Novick and Wiener (29) in which toluene is employed for lysis of the bacteria was routinely used on culture aliquots because of the ease and reproducibility of the method. The effects of an added emulsifier, sodium deoxycholate, on the reproducibility of these assays was found to be negligible, even though its use was recommended by the authors. The toluene-treated bacterial suspension was used as the stock solution for all further assays. The method is described in Appendix II.

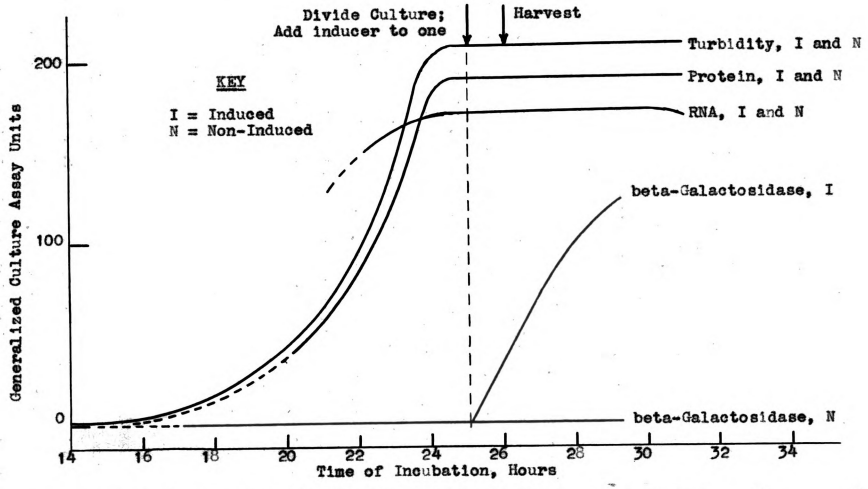

Figure 1.--Gratuitous Induction of beta-Galactosidase in Non-Growing E. coli

The method of Lowry, et al. (30), was used for the protein assay as a means of following culture growth. The detailed procedure used in the determination of the protein concentration in the bacterial culture is also given in Appendix II and the effects of varying the contact time and temperature of the cultures with toluene over wide ranges gave insignificant changes in the values obtained for the protein concentration. The one observed exception resulted from treatment of the culture with toluene over a greatly extended period. As a result, the protein began to coagulate and gave a flocculent precipitate which adversely influenced the readings obtained in the spectrophotometer. Storage of the stock assay solution at 10-20° C. for periods up to one week also resulted in no change in the concentration values of the assayed protein.

The orcinol method of determining ribonucleic acid was used as the third means of following culture growth. The detailed procedure for this assay is also included in Appendix II. In all cases the three criteria, those of turbidity, protein concentration and ribonucleic acid concentration, were satisfactory as a means of estimating levels of culture growth.

In the experiments of Rickenberg and Lester (26) it was found that so-called preferential enzyme synthesis occurred during the period of diauxie of an inducible bacterial culture growing on a limiting amount of glucose but in the presence of a compound such as lactose which serves both as

a beta-galactosidase inducer and substrate. During the period of growth when bulk protein synthesis had ceased and culture turbidity had reached a plateau, there occurred a sudden 1000-fold increase in rate of synthesis of beta-galactosidase over that in non-induced cultures. Once bacterial growthresumed on the new substrate the rate of formation of the new enzyme remained at the high level typical of fully induced cultures.

In order to verify their results in our laboratory and those of another of their experiments in which the gratuitous inducer melibiose was used so that growth could not be resumed, following the growth plateau, several cultures of ML 30 were grown on glucose in the presence of lactose or melibiose as inducers. A modification of the method of Rickenberg and Lester was used routinely for the assay of the induced enzyme beta-galactosidase and is outlined in detail in Appendix II. Its rationale is based on the ability of the enzyme to hydrolyze a variety of substrates having a beta-D-galactosidic linkage. The synthetic substrate o-nitrophenyl-beta-D-galactoside (ONPG) is readily hydrolyzed by the enzyme to yield the yellow substance o-nitrophenol which may then be estimated colorimetrically. Relative enzyme concentrations may be determined in this way under standard conditions of ONPG.

The assay system was examined from several points of view before the specific method was finally chosen. The

spectrum of the chromogen o-nitrophenol was determined and found to present a maximum at 245 mμ in water with a molar extinction coefficient of 3050. In 0.4 M potassium carbonate the maximum is shifted to 420 mμ with a molar extinction coefficient of 4800, agreeing with the findings of Dearden and Forbes (31). The use of potassium carbonate in the assay was therefore settled upon because of its action in stopping the enzymatic reaction, because of the increased sensitivity of the method and because of the convenience of using the Klett-Summerson photo-electric colorimeter with the 420 mμ filter for the assay. Because of the possibility of non-enzymatic hydrolysis of the ONPG by the basic solution in this assay system, several experiments were performed which showed that over reasonable periods of time there was no significant increase in reagent blank values under conditions of increased time of contact of the ONPG with the potassium carbonate or under conditions of varying reagent temperatures. A plot of absorbancy versus concentration of o-nitrophenol under conditions of the assay gave a straight line indicating the validity of Beer's law in this instance.

Application of the assay to the system of Rickenberg and Lester using ML 30 verified that induction of beta-galactosidase in Escherichia coli growing in a glucose medium occurred only after bacterial growth had ceased according to all three of the previously described criteria. Chromatography of the culture medium showed that this point in the growth curve was reached only when the available glucose had been exhausted.

Melibiose as an Inducer

The melibiose used in these studies was purchased from Nutritional Biochemicals Corporation as d (+) melibiose hydrate of unspecified molecular weight. It was determined to be chromatographically pure using a n-butanol: acetic acid: water 40:10:22 (v/v/v) solvent system on paper, and a modified Tollen's reagent spray. The formula weight of the purchased material may be assumed to be 342.3, which is calculated on the basis of a single molecule of the non-hydrated substance 6-(alpha-D-galactosido)-D-glucose. When this compound was used as the beta-galactosidase inducer it was found that the beta-galactosidase concentration increased linearly with time for some hours after the onset of its synthesis, though there was absolutely no detectable increase in culture turbidity, total culture protein, or total culture ribonucleic acid during this period. In other words, by these criteria there were no observable differences between the turbidity, protein and ribonucleic acid content of induced and non-induced cultures. It was found that melibiose itself produced a green color in the orcinol determinations but this was compensated for by subtracting the reading for a melibiose blank run through the procedure from the colorimetric readings obtained for the induced cultures.

The melibiose concentration was varied in one series of induction experiments in order to determine at what point saturating levels of the inducer were reached. A melibiose

concentration of 0.1 per cent was finally chosen and used in all future experiments. This concentration is some two to three times that required for saturation of the induction system as determined by observing for a maximum rate of beta-galactosidase synthesis.

Induction Conditions for the Experimental Cultures

As indicated in the Introduction, it was necessary to produce two cultures of bacteria identical in every particular so that one could be induced and the other serve as a non-induced control. It was found that the easiest way of producing two identical cultures was to grow one culture to the desired point and then divide it in two. A 2 liter culture was grown at a temperature of 30-37° C., held constant for any given experiment until growth was well up on the plateau. One liter of the culture was transferred to a new flask containing 1 gm. of melibiose in 5 ml. of water, and incubation was continued for several hours. At various intervals the cultures were assayed for turbidity, protein, ribonucleic acid and beta-galactosidase. The results, shown in Figure 1, demonstrate that induction can be effectively initiated even after the cultures have ceased to grow. This system was used for all further experiments.

For those experiments in which P^{32} phosphate was to be employed, the labeled phosphate was added to the large culture immediately prior to its division. Thorough shaking

then insured that the distribution of label was identical in the two divided cultures and that any differences in incorporation observed between the two smaller cultures would be caused by the induction process only. In the experiments which follow, the initial bacterial culture was incubated to the growth plateau in a 5 liter Erlenmeyer flask at 37° C., with shaking, where it was allowed to remain for exactly one hour. At this time 2 mc. of P^{32}-labeled phosphate (obtained from Oak Ridge National Laboratories) was added and thoroughly mixed. Half the culture was then transferred to an identical flask containing 1 g. of melibiose dissolved in 5 ml. of water. Incubation of both flasks was continued for one hour longer at which time the flasks were placed in an ice bath in preparation for harvesting the bacteria. All further experimental operations were performed in the cold unless otherwise indicated. The two cultures were carried through all the following procedures separately but simultaneously to minimize variations in data caused by differences in handling techniques.

Fractionation of Cellular Components

Harvesting the Bacteria

The chilled cultures were centrifuged in batches of 360 ml. each in six 250 ml. plastic centrifuge cups, 3 cups per culture, for 10 minutes at 7,500xg. in the International HR-1 refrigerated centrifuge. The clear supernates were retained for plating and counting of their radioactivity in a Nuclear

gas flow proportional counter. A 10 µl. volume of each was plated on an aluminum planchet, dried under an infrared lamp, and counted. Non-radioactive supernatants were discarded. The counting data are given later in Table 1.

Successive batches of culture were centrifuged on top of preceding batches until all the bacteria had been spun into pellets in the centrifuge cups. It was found advisable and very convenient to color code the cups to avoid inadvertant mixing of the induced culture and the non-induced control culture. Red was used routinely for the induced and black for the non-induced cultures throughout these experiments. After completion of the centrifugation the bacteria were collected in one centrifuge cup, thoroughly dispersed in cold 0.01M potassium phosphate buffer having a pH of 7.0 and containing sufficient magnesium chloride to make the solution 0.001 M in this salt, and re-centrifuged. The presence of the magnesium ion in this concentration was shown by Tissieres and Watson (32) to be necessary to prevent the breakdown of the bacterial ribosomes below the 32 S and 51 S particle sizes.

Sonication of Bacteria

The bacteria were finally dispersed in 15 ml. of the same buffer and one culture was transferred to a chilled Raytheon 10 KC sonic disintegrator. Another 10 ml. of buffer was used to rinse the bacteria into the sonicator cup from the centrifuge cup. The bacteria were subjected to this

treatment for 15 minutes at full intensity after which time the bacterial sonicate was transferred into a 50 ml. lusteroid centrifuge tube. The cup was rinsed with 15 ml. of buffer and the washings were combined with the sonicate in the 50 ml. tube. After thorough washing of the sonicator cup the other bacterial culture was sonicated in the same way. Examination of the sonicate by means of a phase contrast microscope showed that in excess of 95 per cent of the cells were disintegrated using this treatment.

Removal of Mitochondrial and Cellular Debris

The sonicated extracts were centrifuged for 10 minutes at 20,000 x g in the HR-1 International refrigerated centrifuge. The opalescent supernatants were carefully removed with 50 ml. pipets leaving the mitochondrial pellets and surface lipids behind.

Preliminary Examination of Deoxyribonucleic Acid

In order to examine the general effects of the melibiose inducer, the relative incorporation of P^{32}-phosphate in the deoxyribonucleic acids of the induced and non-induced cultures was determined. If any greater tendency exists for growth and cell division in the induced culture, this would be expected to result in an increased incorporation of label into the deoxyribonucleic acid of that culture. One-third of the 20,000 x g supernatant from the preceding section was withheld from the ultracentrifugation procedure in preliminary

experiments and was treated with ribonuclease followed by the deproteinization method of Sevag (33) for the isolation of deoxyribonucleic acid. (See Appendix IV.)(The method of Mirsky and Pollister (34), in which the deoxyribonucleic acid is precipitated from a 0.14 sodium chloride solution, was tried in one experiment but yielded no product.) After nine consecutive extractions of the deoxyribonucleic acid solution with the organic solvent it appeared that complete deproteinization had been obtained as indicated by an absence of further sediment at the interface of the two liquids. The layers were separated, one volume of ethanol was added to the aqueous layer, and the deoxyribonucleic acid precipitate was centrifuged at 15,000 x g for 15 minutes. The deoxyribonucleic acid pellet was taken up in 10 ml. of water containing 10 µg. of ribonuclease and was allowed to stand overnight at 4° C. To this solution was added 0.5 gm. of sodium chloride and one volume of ethanol. The resulting precipitate was centrifuged, taken up in 10 ml. of water and dialyzed versus water. The deoxyribonucleic acid solution was centrifuged briefly at 20,000 x g and the supernatant was lyophylized to dryness. The resulting white fluffy material gave a strongly positive Dische, a negative orcinol and a negative biuret test, thereby indicating the presence of deoxyribonucleic acid and the absence of ribonucleic acid and protein as contaminants (see Appendix III).

Both the induced and non-induced samples of deoxyribonucleic acid were then plated in duplicate on tared aluminum

planchets. The planchets were re-weighed and then counted as described. The results of these experiments showed that the incorporation of P^{32}-phosphate into the deoxyribonucleic acid of the induced culture was the same as in the non-induced culture, indicating that no differential growth had occurred between the two and verifying the gratuity of melibiose. These results are included as part of Table 1.

The Ribonucleic Acids

To the remaining two-thirds of the 20,000 x g supernatant in these preliminary experiments was added sufficient 1 X-crystallized deoxyribonuclease (purchased from Worthington Biochemical Corporation) to make the final concentration 1 µg. per ml. The soluble and ribosomal fractions were then obtained by ultracentrifugation.

Ultracentrifugal separation of fractions: The 20,000 x g supernate was routinely centrifuged at 105,000 x g in the Spinco model L ultracentrifuge for 2 hours according to the method of Tissieres and Watson (32). The ultracentrifugal supernatants, designated the soluble fraction, were decanted, combined and saved. The jelly-like ribosomal pellets were transferred with stirring and rinsing to a single tube and were dispersed in 3 ml. of buffer by twirling a metal spatula in the tube. The tube was then filled with buffer and the centrifugation was again performed, this time for one and one-half hours. The pellet was washed in this manner once more, retained, and designated the ribosomal ultracentrifugal

fraction. It was decided to discard the washings rather than to combine them with the soluble fraction after preliminary experiments revealed that some breakdown of ribosomes did seem to occur during prolonged centrifugation.

Phenol preparation of ribosomal ribonucleic acid: The final 105,000 x g pellet was suspended in 5 ml. of 0.02 M potassium phosphate buffer, pH 7, and the ribosomal ribonucleic acid was prepared from this solution by a modification of the phenol procedures of Gierer and Schramm (35) and Kirby (36) (see Appendix IV).

One-tenth volume of a 20 per cent solution of potassium acetate adjusted to a pH of 5 was added to the ribonucleic acid solutions. The ribonucleic acid was precipitated by the addition of two volumes of 95 per cent ethanol in the cold, centrifuged, and re-precipitated as above. The final precipitate was dissolved in 10 ml. of water and dialyzed exhaustively against water. An attempt to use methyl cellosolve to separate the ribonucleic acid from polysaccharides according to the method of Kirby (36) was not successful. After centrifuging the solutions for 20 minutes at 20,000 x g their ultraviolet spectra were obtained in the Carey recording spectrophotometer and appeared to be typical of ribonucleic acid. There were no detectable differences between the induced and the non-induced ribonucleic acid solutions.

The solutions were then lyophylized. The white fluffy material obtained in this way gave a strongly positive orcinol,

a negative Dische and a negative biuret test, thereby demonstrating the presence of ribonucleic acid and the absence of contaminating deoxyribonucleic acid and protein. The ribonucleic acid was plated and counted in the same manner as the deoxyribonucleic acid in the preceding section. A significant increase in the rate of turnover or synthesis of the ribosomal ribonucleic acid was observed as a result of melibiose induction. These results are also summarized in Table 1.

Phenol preparation of soluble ribonucleic acid: The so-called soluble ribonucleic acids were isolated from the supernatants of the ultracentrifugal fractionating procedure described previously by the method of Hoagland, et al. (37), which follows. This fraction of cellular ribonucleic acid is known to contain the species responsible for the transfer of amino acids to the ribosomes.

The supernatant fraction was adjusted to a pH of 5.2 by the careful addition of acetic acid. The resulting precipitate was centrifuged at 20,000 x g for 10 minutes, redissolved in water, and again precipitated by adjusting the solution to a pH of 5.2 with acetic acid. Following centrifugation, the precipitate was dissolved in potassium phosphate buffer having a pH of 7.0. The protein and deoxyribonucleic acid were then removed by the phenol method as described in Appendix IV.

The soluble ribonucleic acid was isolated and purified in the same manner as the ribosomal ribonucleic acid in the

preceding section and was similarly dialyzed, lyophylized, characterized, plated and counted. The results of these radioactive determinations indicated in a manner similar to that seen for the ribosomal ribonucleic acid that there was a marked effect of induction on the metabolism of soluble ribonucleic acid in these non-growing cultures. These results are given in detail in Table 1.

Table 1.--Relative Incorporation of P^{32}-Phosphate into Cell Constituents of Induced and Non-Induced E. Coli*; Preliminary Experiments

	Total Counts Per Minute		
Fraction	Induced	Non-Induced	Ratio of I/N
Culture Supernatants	1.48×10^9	1.47×10^9	1.01
Deoxyribonucleic acid	3.86×10^3	4.24×10^3	0.97
Ribosomal Ribonucleic acid	11.1×10^3	8.93×10^3	1.24
Soluble Ribonucleic acid	16.6×10^3	12.7×10^3	1.31

*Grown on high-phosphate medium of Monod, et al.

Labeling Patterns Among Molecular Components

The preceding preliminary data indicated that there may be a specific and very pronounced effect of induction on the metabolism of both the ribosomal and soluble fractions of the ribonucleic acids. The following experiments were performed in order to examine in more detail the effects of induction on the molecular components of the bacterial cells.

Examination of the lipid fraction was undertaken with the intention of obtaining a further check on the specificity of the effects of induction; examination of the ribonucleotides was undertaken to allow a precise evaluation of the nature of the variations in ribonucleic acid metabolism already observed; and examination of the deoxyribonucleic acids was repeated using a new procedure as a check on the data obtained in the preceding experiments.

General

A modification of the method of Volkin and Astrachan (28) was used for the sequential isolation and analysis of lipids, ribonucleic acid and deoxyribonucleic acid from the centrifugal fractions previously described. The incorporation of P^{32} into such fractions obtained from induced and non-induced cultures is compared in Table 2, page 31.

In this procedure the lipids and proteins were first removed and the ribonucleic acids were then subjected to base hydrolysis in the presence of the deoxyribonucleic acid. The resulting mixture of nucleotides and deoxyribonucleic acid was placed on an anion exchange column. The nucleotides were eluted sequentially from the column and the deoxyribonucleic acid was finally extracted with strong acid from the resin. The incorporation of P^{32} in the induced and non-induced cultures was then compared, not only in the lipid and deoxyribonucleic acid fractions, but also in the ribonucleotides of the ribonucleic acids themselves. This procedure was followed to determine whether the observed difference

Table 2.--Relative Incorporation of P^{32}-Phosphate into Cell Constituents of Induced and Non-Induced *E. coli**; Complete Experiments

Fraction	Total Counts Per Minute		
	Induced	Non-Induced	Ratio of I/N
Culture Supernatants	1.97×10^9	2.05×10^9	0.96
Deoxyribonucleic acid	36.3×10^3	35.6×10^3	1.02
Ribosomal Ribonucleic acid	90.6×10^3	70.3×10^3	1.29
Soluble Ribonucleic acid	$201. \times 10^3$	$152. \times 10^3$	1.32
Ribosomal Lipids	$198. \times 10^3$	$160. \times 10^3$	1.24
Soluble Lipids	36.1×10^3	28.4×10^3	1.27

*Grown on low-phosphate medium of Volkin and Astrachan.

actually resided in the ribonucleic acids or in undetected contaminants of the ribonucleic acids as they were isolated in the earlier experiments. Detailed analysis of the nucleotides was then performed to determine the percentage composition of the bulk ribonucleic acid isolated and, in radioactive experiments, of the newly synthesized ribonucleic acid.

Isolation of Lipids

To each of the supernatant ultracentrifugal fractions obtained as described earlier was added 0.04 volumes of reagent grade 70 per cent perchloric acid. The ribosomal fraction was either taken up in water and handled similarly,

or introduced into the procedure at the point of addition of ammonium acetate.

The precipitated acid insoluble material was isolated by centrifugation and neutralized by the addition of 1 ml. of 1 M ammonium acetate. The material was transferred to a 50 ml. round bottom flask fitted with a water-cooled condenser having a standard taper ground glass joint, and refluxed for 30 minutes on a hot plate with 20 ml. of ethanol: ethyl ether, 3:1 (v/v).

The solid residue was removed from the organic solvent by gentle vacuum filtration through a fine fritted glass filter. The flask was rinsed twice with 10 ml. of fresh solvent and this was also passed through the filter. The filtrate was then made up to 50 ml. in a volumetric flask, 0.1 ml. aliquots from each were plated in duplicate on aluminum planchets, dried gently under an infrared lamp, and the activity from each planchet was measured as previously described. The measured radioactivity was then related to the total phosphorous content of the extracts by means of an organic phosphate determination. An aliquot of the lipid extract was transferred to a digestion tube after which the organic solvent was evaporated by a stream of air under a heatlamp. The dry residue was digested by heating with concentrated sulfuric acid after which the total phosphate was determined by a modification of the colorimetric method of King (see Appendix III). The total incorporation of P^{32} into the induced and non-induced lipid fractions are compared

in Table 2.

Solubilization and Isolation of the Nucleic Acids

The solid residues of the soluble and ribosomal centrifugal fractions remaining after the extraction of lipids were suspended in 10 ml. of 0.1 M Tris buffer, pH 7.8: 2 M potassium chloride, 1:9 (v/v) and placed in a boiling water bath for 1 hour. The suspensions were occasionally swirled or stirred to insure uniform dispersion of the solids. The suspensions were then cooled and filtered under vacuum through a sintered glass filter of medium porosity. The proteins were retained on the filter.

The protein residue was washed with 5 ml. of fresh Tris-buffered potassium chloride solution and the clear filtrates, which contained the solubilized nucleic acids, were combined in 150 ml. beakers and chilled. Two and one-half volumes of cold 95 per cent ethanol were added and the nucleic acids were precipitated at -10° C. The sediment of nucleic acids was centrifuged in the cold room in a clinical centrifuge, redissolved in 10 ml. of the Tris-potassium chloride solution and re-precipitated in the cold by the addition of two and one-half volumes of ethanol.

After centrifugation the nucleic acids were taken up in 10 ml. of water and dialyzed exhaustively versus water in the cold. The absorbancy of the dialysate at 260 mμ was measured and found to be the same as that of water.

Hydrolysis of Ribonucleic Acid

The dialyzed solutions of nucleic acids were transferred to 50 ml. beakers. One-tenth volume of 1 N potassium hydroxide was added to each beaker. The beakers were covered with aluminum foil or "parafilm" to retard evaporation and placed in an oven at 37° C. for 16 hours. The resulting alkaline solutions contained deoxyribonucleic acid and a mixture of the 2' and 3' isomers of the ribonucleotides resulting from the degradation of the ribonucleic acid. The solutions were diluted at least ten times to reduce the anion concentration below 0.01 M, and adjusted to pH 8-10 with hydrochloric acid in preparation for anion exchange chromatography.

Anion-Exchange Chromatography of Ribonucleotides

General: The procedure used is a modification of the method of Cohn (38) in which the ribonucleotides are absorbed onto a column of Dowex-1 resin (chloride form) and eluted in the sequence cytidylic, adenylic, uridylic and guanylic acids by a succession of increasingly concentrated solutions of hydrochloric acid.

Several problems arose in the initial nucleotide separations. Difficulties were experienced in obtaining complete separation of the cytidylic and adenylic acids and there seemed to be an excessively large dead volume of eluant before the first peak appeared. These difficulties were completely remedied by exercising exact pH control of the eluants by

means of a Beckman pH meter rather than using simple dilution of a concentrated solution of hydrochloric acid to adjust normality.

A second problem appeared when it was found that the nucleotides were being eluted from the columns in such low concentrations that identification of the nucleotides, estimation of their concentrations and determination of their radioactivity was very difficult. The problem, which was present because of the small quantities of ribonucleic acid obtained from 1 liter of bacterial culture, was solved by reducing the cross-sectional area of the columns and the flow rate of the eluant each by a factor of three. Graduated 5 ml. pipettes plugged with glass wool were used for the columns and a flow rate of 1/3 ml. per minute was found to give optimum separation with highest nucleotide concentrations and eluant flow rates.

The anion exchange columns were prepared in identical pairs and were handled as identically as possible throughout the chromatographic procedures. The induced sample was applied to one column and the corresponding non-induced control sample was applied to the other. The ribosomal ribonucleic acid hydrolysates were generally chromatographed first and the soluble ribonucleic acid hydrolysates later since the specific activities of the soluble samples were much higher and could tolerate a greater decay time until being counted.

Procedure: Dowex-1 resin, X8 cross linkage and 100-200 mesh was washed in 1 N hydrochloric acid, rinsed with water, and poured onto the columns in a slurry until the bed volumes were approximately 5 ml. each as read on the graduations etched on the columns. The columns were washed with 1 N hydrochloric acid, water, 0.01 N sodium hydroxide, water, 1 N hydrochloric acid and water successively. The absorbancy at 260 mμ of the final water wash was followed in the Beckman DU spectrophotometer versus a water blank until it reached a value near zero.

At this time the nucleotide solutions, previously prepared for chromatography as described, were passed through the columns. The immediate effluents were collected and measured for absorbancy at 260 mμ. These values were generally very low, indicating that absorption of the nucleotides on the columns had been complete. A water wash followed which again was collected and also had a negligible absorbancy at 260 mμ. When the absorbancy reached base-line, elution with various solutions of hydrochloric acid was begun.

The initial elution was accomplished at a flow rate of 1/3 ml. per minute with hydrochloric acid having a pH of 2.65. An automatic fraction collector was used to collect 5 ml. fractions until the first UV absorbing material, consisting of cytidine 2' and 3' phosphates, had been eluted. By following the elution in the Beckman DU it was readily apparent

when this point was reached. Readings were taken at 250, 260, 280, and 290 mμ for all tubes so that positive identification of the peaks of UV-absorbing compounds could be ascertained by a comparison of absorbancy ratios at the various frequencies with the data of Volkin and Cohn (39). A summary of their applicable data is included in Appendix V.

Following the complete elution of the very sharp cytidylic acid peak, which usually occurred in the first 100 ml., the fraction collector was adjusted to collect 20 ml. per fraction for the remainder of the chromatographic run. The double peak of the 2' and 3' adenylic acid isomers generally came off in less than 400 ml., after which time the eluant was changed to a solution of hycrochloric acid having a pH of 2.45. The single broad peak which followed contained the 2' and 3' uridylic acid isomers. This peak, which again required less than 400 ml. of eluant, was followed by a final change of eluant to a hydrochloric acid solution having a pH of 2.2.

The double peak representing the 2' and 3' isomers of guanylic acid which next appeared also required less than 400 ml. of eluant for its complete removal. Elution was continued until the absorbancy at 260 mμ was not significantly above base-line. Only the four classical peaks were obtained in significant amounts.

Criteria of Purity of Eluted Ribonucleotides: The purity of the nucleotides under each peak was established by several

criteria. The ratios of absorbancy at 280 mμ and 260 mμ were calculated for each fraction from the measured absorbancies and compared with the values of Volkin and Cohn given in Appendix V. Except in isolated cases the ratios agreed closely with these values.

The second criterion concerned the radiochemical purity of the fraction within each peak. Two milliliter aliquots of each fraction were dried on platinum planchets and counted in a flow detector as previously described. The speicific activities of each fraction in counts per minute per millimole were calculated using the net counts per minute, the absorbancy of each fraction at 260 mμ, the molar extinction coefficients of Volkin and Cohn given in Appendix V, and the plated volume. The activity measurements for each planchet were normalized to one given time for each experiment by making decay rate corrections. This time was always set as the time at which the experiment was begun.

It was found that each of the fractions under any particular peak had the same specific activity. In other words, the peaks of radioactivity being eluted from the column coincided precisely with the nucleotide peaks as determined and identified spectrophotometrically. These findings assured the radiopurity of the nucleotides obtained from the columns. In those cases where partial separation of the 2' and 3' isomers occurred, it was found that the specific activities of the two isomers were identical, as expected.

Nucleotide ratios of eluted ribonucleotides: Once the purity of the eluted nucleotides had been established, further experiments were performed to determine the molar ratios of the ribonucleotides thus eluted. The resulting ratios represent the nucleotide ratios of the parent bulk ribonucleic acids that had been hydrolyzed to yield the nucleotides. In these experiments the fractions under each nucleotide peak were pooled and the combined volume was measured. The absorbancy of the solution was determined at 260 mμ and the total number of moles of nucleotide present in the solution was calculated by means of the extinction coefficients given in Appendix V. The molar percentage of each nucleotide derived from the bulk ribonucleic acid of each chromatographed sample was then readily obtained. The data for the bulk ribonucleic acid of the induced and non-induced cultures are given in Table 3. No changes in the amounts of bulk ribonucleic acid were anticipated or found in the non-growing cultures. The small differences observed between the proportions of the nucleotides of the two cultures, therefore, give a measure of the experimental error encountered in these measurements. The averages of the induced and non-induced values were taken as probably being more accurate than the individual determinations.

See Table 3 on page 40.

Nucleotide ratios of newly synthesized ribonucleic acid: If it is assumed that the source of any newly-formed ribonucleic acid is a uniformly labeled pool of radioactive

Table 3.--Percent Nucleotide Composition of Bulk RNA From Induced (I) and Non-Induced (N) Cultures

	*Bulk Ribosomal RNA			*Bulk Soluble RNA		
Nucleotide	I	N	Avg.	I	N	Avg.
Cytidylic acid	20.7	20.9	20.8	26.2	26.8	26.5
Adenylic acid	24.0	24.2	24.1	21.9	21.5	21.7
Uridylic acid	20.5	19.7	20.1	18.5	18.7	18.6
Guanylic acid	34.8	35.2	35.0	33.4	33.0	33.2

*Calculated from absorbancy data.

precursors, then the total amount of radioactivity found in each of the nucleotides after hydrolysis of the ribonucleic acid should be in proportion to the amount of this particular nucleotide introduced into the newly-formed ribonucleic acid. Based on this assumption and a knowledge of the total activity found in each nucleotide it is possible to calculate the nucleotide ratios of the ribonucleic acid synthesized after the introduction of the labeled phosphate; that is, after the onset of induction in these experiments. A comparison of these values for the induced and non-induced cultures may then give an insight into the detailed effects of induction on the metabolism of the ribonucleic acid fractions.

To obtain accurately the total counts present in each of the nucleotides, it was necessary to determine accurately the molar specific activities of the individual nucleotides and multiply this figure by the total moles of the particular nucleotide as obtained in the preceding section. To this end the nucleotide solutions were concentrated on an auxiliary

column of Dowex-1. A 10 ml. buret was plugged with glass wool and layered to a height of 2 cm. with resin in the same manner as was used for preparation of the chromatographic columns. The resin was similarly treated in preparation for absorption of the nucleotides. The dilute nucleotide solution was then adjusted to a pH of 8-10 with aqueous ammonia and passed through the buret. The column was rinsed with water until the absorbancy at 260 mμ was base-line and the nucleotides were eluted with an aqueous solution of hydrochloric acid at a pH of 2. Three-milliliter fractions were collected until the greater portion of the nucleotide had been removed. The absorbancies of the eluants were measured at 260 mμ and 280 mμ and the ratios were calculated to insure the purity of the concentrated nucleotide. The columns were regenerated by washing with 1 N hydrochloric acid to base-line, and then water. One-tenth milliliter aliquots of the nucleotide were plated and counted on aluminum planchets as described. After normalizing the counts obtained to the time of the start of the experiment, the specific activities of the plated nucleotides were readily calculated as previously described. These values were then multiplied by the total amount of the nucleotide isolated, as determined by absorbancy data in the preceding section, to obtain the total activities of each nucleotide. The ratios of these total activities were then calculated. Values obtained in these experiments are given in Table 4.

Table 4.--Percent Nucleotide Composition of Newly-Formed Ribonucleic Acid From Induced (I) and Non-Induced (N) Cultures Calculated From P^{32}-Phosphate Incorporation Data

Ribosomal RNA Nucleotides	Moles of Nucleotide	Specific Activity CPM/Mole	Total CPM	% Composition Based on Total CPM	Average of I and N % Comp.
I-CA	2.09×10^{-3}	12.5×10^{6}	26.1×10^{3}	19.2	19.6 CA
I-AA	2.43	16.7	40.5	29.8	28.8 AA
I-UA	2.07	13.1	27.2	20.0	20.5 UA
I-GA	3.52	12.0	42.2	31.1	31.1 GA
N-CA	*1.38×10^{-3}	11.2×10^{6}	15.5×10^{3}	20.0	
N-AA	1.60	12.5	21.5	27.8	
N-UA	1.30	12.5	16.3	21.0	
N-GA	2.33	11.0	24.2	31.2	
Soluble RNA Nucleotides					
I-CA	1.31×10^{-3}	34.1×10^{6}	44.7×10^{3}	26.4	26.8 CA
I-AA	1.10	39.6	43.5	25.7	25.4 AA
I-UA	0.93	34.8	32.4	19.1	19.9 UA
I-GA	1.67	29.0	48.5	28.6	28.0 GA
N-CA	*0.87×10^{-3}	26.0×10^{6}	22.6×10^{3}	27.1	
N-AA	0.70	29.7	20.8	25.0	
N-UA	0.61	28.4	17.3	20.7	
N-GA	1.07	21.3	22.8	27.3	

*These values are low because of accidental loss of approximately 1/3 of the non-induced culture during sonication.

Recovery of Deoxyribonucleic Acid

The anion exchange column used in the separation of ribonucleotides retains the deoxyribonucleic acid throughout the elution procedure. The resin was removed from the column after completion of each chromatogram and heated on a steam bath for 10 minutes with approximately 2 volumes of 5 N hydrochloric acid.

The solution, which then contained the degraded deoxyribonucleic acid, was decanted or filtered off and the resin was rinsed several times with acid. A fritted glass filter was used for separation of resin and the acid solution. The washed resin was found to be free of radioactivity and was used to make up new anion exchange columns. The combined filtrates were diluted to 100-200 ml. in a volumetric flask of the proper size. The spectrum of each sample was determined in the Carey recording spectrophotometer and the absorbancies at 260 mμ were measured in the Beckman DU. It was found that only 5-10 per cent of the ultraviolet absorbing material obtained in this way was in the ribosomal ultracentrifugal fractions, whereas the remainder was obtained from the soluble fractions as would be expected for bacterial deoxyribonucleic acid. The spectra of the material obtained from the soluble fractions showed the typical maxima and minima of nucleic acids, but the small amount obtained from the ribosomal fractions showed considerable contamination by some material, probably protein, which exhibitied strong absorption between 230 and 250 mμ.

Two milliliter aliquots of the deoxyribonucleic acid solutions were plated in duplicate on platinum planchets, dried and counted as previously described. To compare specific activities the average counts per minute were normalized to a specific time for the samples and divided by the absorbancy at 260 mμ. The results of these experiments are given in Table 2. A comparison of both these specific and total activities of the deoxyribonucleic acid of the induced and non-induced soluble fractions confirm the results obtained previously with deoxyribonucleic acid obtained by the Sevag method. The ribosomal deoxyribonucleic acid showed wide variations from run to run thereby confirming its impurity as was indicated by an examination of its spectrum.

Effects of 5-Hydroxyuridine on the Metabolism of Ribonucleic Acid During Induction

As was discussed in the introduction to this dissertation, the compound 5-hydroxyuridine has the ability even in very low concentrations to inhibit markedly the induced synthesis of beta-galactosidase in exponentially growing *E. coli*. In such experiments it does not exert apparent effect on the over-all rate of protein synthesis of the culture (23). This inhibition was reported by these workers to occur even when the synthesis of beta-galactosidase was proceeding at a maximal rate. This inhibitory effect has been cited as presumptive evidence for the requirement of prior or concomitant synthesis of at least some ribonucleic acid

during enzyme synthesis. It was therefore of considerable interest to try to locate the site of action of the uridine analog by introducing it into the non-growing system described in the preceding experiments.

A sample of 5-hydroxyuridine was purchased from Nutritional Biochemicals Corporation for these experiments. The initial experiments demonstrated that the amount of 5-hydroxyuridine quoted by Spiegelman, et al. (23), as being sufficient to produce complete inhibition in growing cultures had absolutely no effect in the non-growing system used here. The quoted concentration of 5 µg. per ml. was increased to 500 µg. per ml. in another series of experiments and the effect on the rate of synthesis of beta-galactosidase in the induced cultures was still found to be negligible. This line of experimentation was therefore regretfully abandoned.

Chitosan Chromatography of Undegraded P^{32}-Labeled Ribonucleic Acid

Preparation of Undegraded P^{32}-Labeled Ribonucleic Acid

Induced and non-induced cultures of E. coli were grown and labeled with P^{32}-phosphate just prior to induction by the techniques already described. The bacteria were harvested and sonicated also as described. Following sonication, 1 µg. per ml. of deoxyribonuclease was added to the culture extracts and the soluble and ribosomal ultracentrifugal fractions were prepared as before. The ribosomal pellet was dispersed in

30 ml. of the same buffer used in these centrifugations and 30 mg. of sodium dodecyl sufate ("Duponol" C) was dissolved in the solution to enhance separation of the ribosomal protein from the ribonucleic acid.

The ribonucleic acids of the soluble and ribosomal fractions were prepared for these chromatographic separations by the phenol procedure described in Appendix IV.

One-tenth volume of a 20 per cent solution of potassium acetate adjusted to a pH of 5 was added to the ribonucleic acid solutions. The ribonucleic acid was precipitated by the addition of two volumes of 95 per cent ethanol in the cold, centrifuged, and re-precipitated as above. The final precipitate was dissolved in 10 ml. of water and dialyzed exhaustively against water.

The ribonucleic acid solutions were mixed after dialysis with 0.1 volume of 0.1 M potassium phosphate buffer having a pH of 6.5 in preparation for absorption on the chitosan column. The solutions were allowed to warm to room temperature so that dissolved gases would be forced out of solution before they were placed on the columns.

Preparation of the Columns

A personal communication from Dr. Herbert Pahl, currently with the National Institutes of Health, disclosed that he had been able to obtain a number of discrete fractions of ribonucleic acid by chromatography on a column of cellulose coated with chitosan (deacetylated chitin) obtained from Mann Research

Laboratories, Incorporated. He was kind enough to provide complete details for preparation of the column and appropriate schedules of elution. However, a modification of the column size and elution schedules was found necessary for these experiments and is described in the following paragraphs.

Sufficient exchanger material for two columns was prepared by suspending 2 g. of cellulose in 30 ml. of chitosan solution. The chitosan solution is stable and may be prepared in advance and stored. To a desired volume of 1 per cent acetic acid was added 1 mg. of chitosan per ml. of liquid. Solution was easily effected with a magnetic stirrer and was carried out at room temperature. To the cellulose suspension was added an additional 20 ml. of 1 per cent acetic acid while stirring vigorously with a magnetic stirrer. To this suspension was then added dropwise over a period of 10-30 minutes a solution of potassium hydroxide until the pH reached a value of 9-9.5.

The exchanger was transferred to a pair of identical columns made by plugging two graduated 10 ml. pipettes with glass wool. Exchanger was poured into each column until the exchanger bed was approximately 9 cm. in length. The column was then washed with 0.01 M potassium phosphate buffer adjusted to a pH of 6.2-6.5, until the absorbancy of the eluate was base-line at 260 mμ.

Elution Schedule for Ribonucleic Acid Chromatography

The solutions of phenol-prepared ribonucleic acid were next passed through the column. The eluates were collected and the absorbancy at 260 mμ measured to insure that complete absorption actually occurred. The column was again washed with buffer previously adjusted to a pH of 6.2-6.5 until the eluate absorbancy reached base-line.

Elution of the ribonucleic acid was initially attempted using a gradient elution formula recommended by Dr. Pahl. It was soon found that by far the greater portion of the ribonucleic acid remained tightly bound to the exchanger with the recommended schedule. Various attempts were made to devise either a continuous or a discontinuous elution sequence that would meet the requirements of reproducibility and completeness of elution. The discontinuous system described below was selected as being the most satisfactory.

Eluant	Schedule
1.	Elute with 0.1 M phosphate, pH 7.0
2.	" " " " " " 7.3
3.	" " " " phosphate/0.5 M naCl adjusted to pH 7.3
4.	" " " " " /1.0 M " " " " " "
5.	" " " " " / " " " " " " 8.0
6.	" " " " " / " " " " " " 9.0
7.	" " " " " / " " " " " " 10.5
8.	" " " " " / " " " " " " 12.0

The effluents were collected on an automatic fraction collector in 15 ml. fractions and the absorbancy of the fractions

was followed at 260 mμ in the Beckman DU. Each eluant was discontinued and the next was begun in its place when it appeared that little additional UV-absorbing material would be eluted with its continued use. This schedule was found to remove over 90 per cent of the soluble ribonucleic acid initially absorbed on the columns, and only some 50 per cent of the ribosomal ribonucleic acid as determined by the fraction of total radioactivity recovered from this column. Because of the likelihood of hydrolysis of the ribonucleic acid in more basic eluants, and the obvious discoloration of the column that attended extended contact with such eluants, the chromatography was terminated as described. The chitosan-cellulose exchanger was replaced for each new sample to be chromatographed.

Analysis of Eluted Ribonucleic Acid

A first approximation of the radiochemical purity of the eluted material was obtained by comparing the absorbancy at 260 mμ for each fraction with the radioactive counts in that fraction. This was done by plating 0.1 ml. of each fraction on an aluminum planchet, drying under an infrared lamp, and counting in a Nuclear proportional gas flow detector as previously described. It was observed that the radioactive peaks coincided precisely with the absorbancy peaks, although they were not of precisely proportional height, indicating that the radioactivity was associated strictly with the UV-absorbing material being eluted. The spectrum of each fraction

containing a significant absorbancy at 260 mμ was then obtained on the Carey recording spectrophotometer and the resulting curves appeared to be similar and in every way typical of ribonucleic acid. They were found to give a positive orcinol and a negative Dische reaction, indicating freedom of the ribonucleic acid from contamination by deoxyribonucleic acid.

In initial experiments the fractions containing the bulk of the radioactive and UV-absorbing materials were dialyzed separately against water until a negative test was obtained for chloride using a silver nitrate solution. The fractions were then measured for absorbancy at 260 mμ and 0.1 ml. aliquots were plated and counted as previously described. After correcting the counts to a specific time for radioactive decay, the relative activities of the fractions were calculated by dividing the counts per minute obtained by the absorbancy values at 260 mμ. These ratios varied somewhat within each peak but the variations were not reproducible from one experiment to the next. The spectra of the dialyzed fractions were again obtained and again appeared to be typical of ribonucleic acid. The absorbancy of the dialysate at 260 mu was also measured and found to be negligible.

To the remainder of the dialyzed fractions was added 0.1 volume of 0.1 M phosphate buffer, pH 6.5. Each fraction was re-adsorbed onto a fresh column, washed with water, and re-chromatographed. The procedure used was to begin the

elution with the eluant prior to the one which caused the peak to be removed from the column initially. In each case very little material was eluted with this initial eluant and the bulk of the material was quickly removed from the column with the next. The fact that the eluted ribonucleic acid fractions re-chromatograph at the same point in the elution sequence is a strong indication that the separation obtained in these chromatograms is real. Figures 2 and 3 show the chromatographic profile of the soluble and ribosomal ribonucleic acids. Absorbancy at 260 mμ is plotted against the fractions collected. The values for absorbancy were calculated from samples diluted by a factor of 10:1 in those cases where the absorbancy of the undiluted fraction exceeded 2.000 at 260 mμ. For purposes of correlating the peaks drawn in the figures with the text, numbers will be assigned to the peaks corresponding to the particular eluant used in the elution of the material obtained.

In later experiments the fractions collected under any one peak were pooled following dialysis and before determining specific activities since the values so obtained were found to be quite reproducible in contrast to the specific activities found for the individual fractions. The use of absorbancy readings at 260 mμ for determination of specific activities was open to some question because of the possibility that differences in absorbancies at 260 mμ could as easily reflect variations in nucleotide content of the ribonucleic acid as differences in actual ribonucleic acid concentrations in the eluted material.

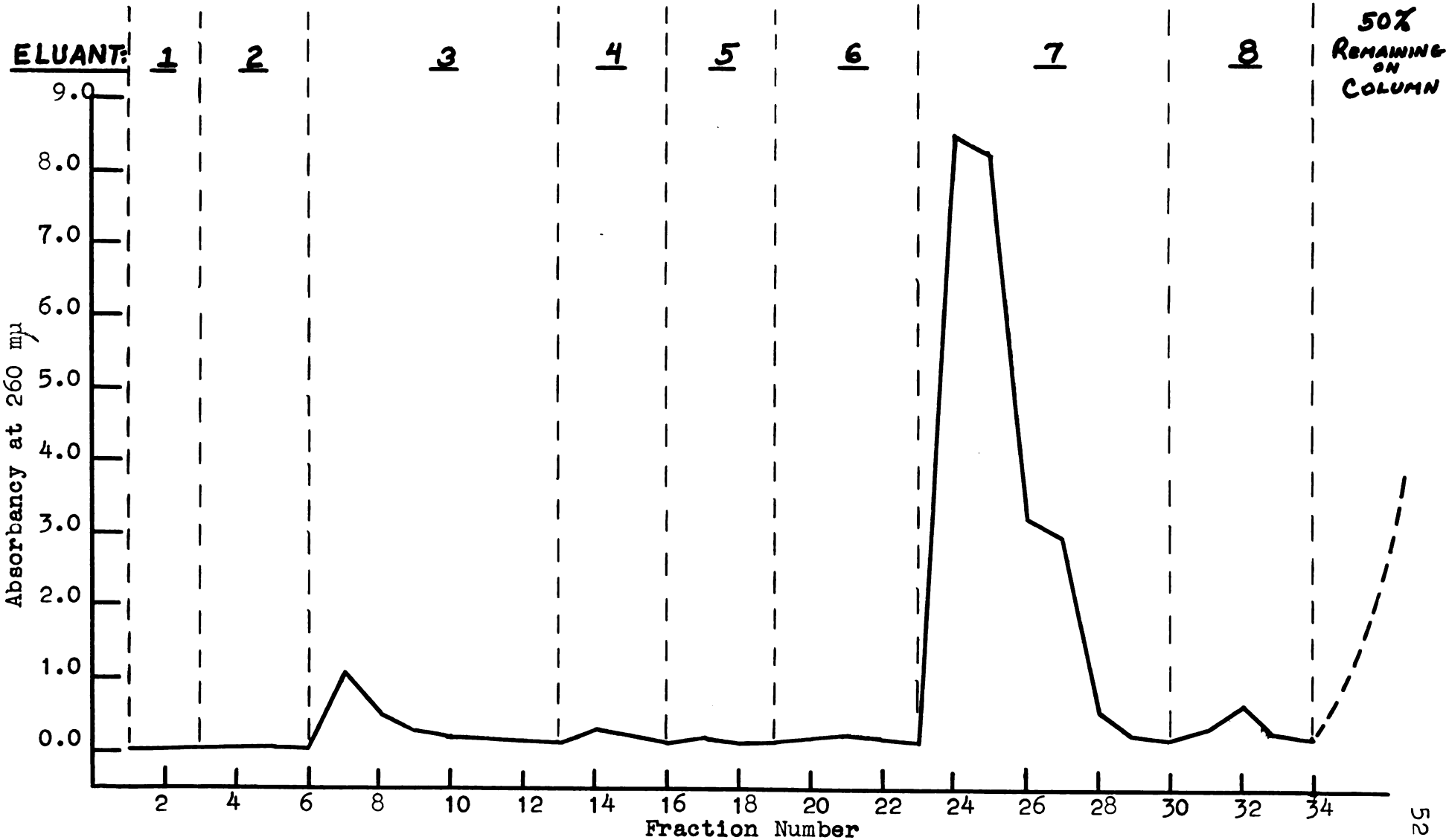

Figure 2.--Fractionation of Ribosomal RNA by Discontinuous Elution from a Chitosan-Cellulose Column

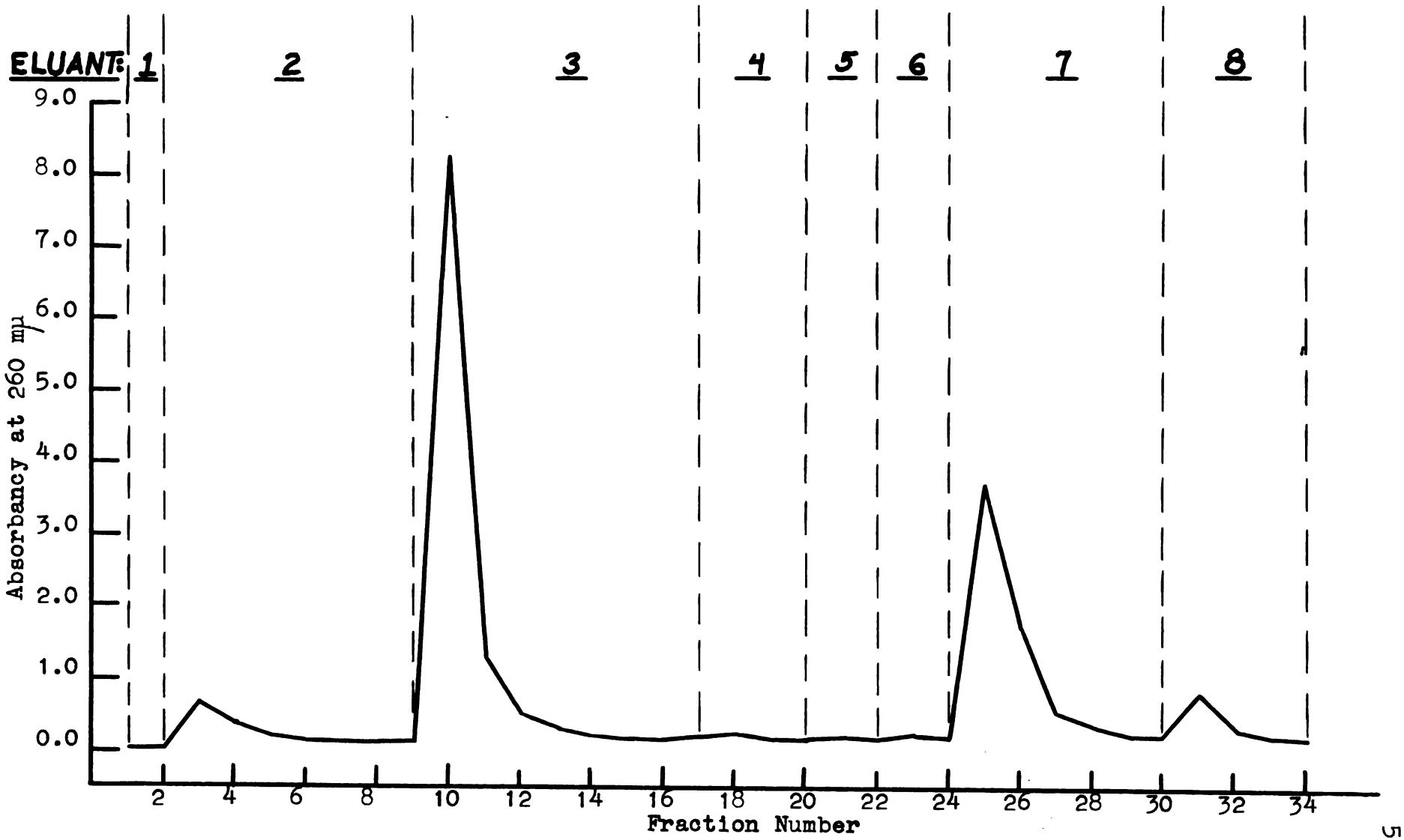

Figure 3.--Fractionation of Soluble RNA by Discontinuous Elution from a Chitosan-Cellulose Column

To insure that the absorbancies at 260 mμ were probably an accurate means of estimating the ribonucleic acid concentrations the absorbancies of each of the pooled fractions were measured at 250, 260, 280, and 290 mμ and the ratios were calculated and compared. Since the ratios were nearly the same for all fractions, indicating that the overall base ratios of their constituent nucleotides are probably quite similar, it may be expected that no great shift in spectral characteristics were present which would make the absorbancy method as a means of estimating ribonucleic acid concentration suspect in this case.

A comparison of the specific activities of the two major and three minor peaks obtained from the ribosomal ribonucleic acid showed that nearly all of the melibiose-stimulated incorporation of P^{32} into the ribonucleic acid of this fraction must have occurred in the material contained in peak 7. By subtracting the amount of UV-absorbing material eluted from the column from the amount initially absorbed, it was apparent that approximately 50 per cent of the ribonucleic acid remained on the column. By subtracting the total counts eluted as compared with the number placed on the column, it was also possible to determine the activity, and hence the specific activity, of the absorbed material remaining. The metabolic turnover in this fraction caused by induction was found to be very low as indicated in Table 5, page 55.

On the other hand, it was found that peak 7 of the soluble ribonucleic acid fractions chromatographed underwent the least metabolic turnover of any eluted from the column. Both

peaks 3 and 8 showed some activity, and peak 2, presumably containing the smallest molecules, had a considerably greater incorporation in the induced relative to the non-induced cultures than any of the other soluble peaks. These results are also summarized in Table 5 below.

Table 5.--Results of Chromatography of Ribosomal and Soluble Ribonucleic Acid on Chitosan Columns-- Specific Activities of Major Fractions of Ribosomal and Soluble Ribonucleic Acid

Chromatographed Sample	CPM/A_{260}			
	Fraction	I	N	I/N
Ribosomal RNA	Total RNA Applied	880	765	1.15
	3	1003	897	1.12
	4	808	740	1.09
	7	770	557	1.38
	8	620	591	1.05
	RNA Remaining on Column	1000	975	1.03
Soluble RNA	Total RNA Applied	1490	1330	1.12
	2	1230	995	1.24
	3	1330	1140	1.17
	7	1670	1570	1.06
	8	1160	1005	1.15

DISCUSSION

DISCUSSION

The increased incorporation of P^{32}-phosphate into the lipid as well as the ribonucleic acid fractions of Escherichia coli during induction with melibiose indicates that the effects of this inducer may be much more general than previously recognized. Halvorson (46) has expressed similar sentiments with respect to this inducer, and it should be recognized that any conclusions drawn from its use must be examined with this point in mind.

The strict gratuity of melibiose is supported by several data obtained in these experiments. Of these the most convincing is the complete lack of effect melibiose has on the rate of turnover or synthesis of the bacterial deoxyribonucleic acid as measured by the rate of P^{32}-phosphate incorporation. If the data of McFall and Stent (47) are valid it would appear that the synthesis of deoxyribonucleic acid is continuous throughout the growing period of a bacterium and is not restricted to a specific stage in the growth cycle related to cellular division. It might, therefore, be expected that if melibiose caused any growth of the bacteria in a general sense, the induced culture should have exhibited an increased incorporation of label in the deoxyribonucleic acid fraction. This increased incorporation did not occur

even though some residual deoxyribonucleic acid turnover or synthesis does occur in non-growing cultures as indicated by the amount of label found in this fraction in the absence of inducer.

The gratuity of melibiose is also supported by the data obtained from the assays on culture protein and ribonucleic acid and from the culture turbidity measurements. The fact that there was no detectable increase in total protein in the culture or of total ribonucleic acid indicates that any changes imposed by the melibiose on cellular metabolism must be subtle ones, not related to the possibility of melibiose serving as a carbon and energy source for sustained growth of the bacteria. The turbidity measurements confirm these conclusions since it is apparent that on the growth plateau following the exhaustion of glucose the bacteria have attained a stable shape and size and that introduction of melibiose into the culture medium has no measurable influence on this stability.

It is only when the very sensitive method of radioactive isotope incorporation is employed that there is any reason to doubt the gratuity of melibiose. If the increased incorporation observed in the lipids and in the ribonucleic acids of both the ribosomal and soluble cellular fractions is directly related to the induction of the beta-galactosidase synthesizing system, then the inducer may still be considered strictly gratuitous. If, on the other hand, some or all of this increased incorporation is not specifically

related to induced enzyme synthesis, then the inducer may not be considered to be strictly gratuitous. Any or all of the observed effects might then be the result of a general stimulation of cellular metabolism, although deoxyribonucleic acid and some chitosan-chromatographed fractions of ribonucleic acid at least seem to be relatively exempt from this stimulation. However, even though the question of the gratuity of melibiose cannot be answered on the basis of these experiments, it should be realized that even in the possible event of the non-gratuity of melibiose, its stimulatory effects appear to be quite selective and may yet be involved primarily with the synthesis and turnover of cellular protein, exhibiting as a secondary effect the induction of the beta-galactosidase system. In this event the labeling data would be related to the synthesis or turnover of protein in general rather than to the single enzyme producing system generally associated with melibiose.

The data of Mandelstam and Halvorson (48), and Mandelstam (49), concerning the turnover of soluble and ribosomal constituents of Escherichia coli under non-growing conditions, indicate that the rate of protein breakdown and resynthesis is approximately 5 per cent per hour in both fractions. Similar figures were obtained for the ribonucleic acids obtained from these fractions. These data demonstrate the probable existence of a dynamic state for many molecular species in non-growing bacteria, and it is possible that it is a small portion of this turnover of protein and ribonucleic

acids that is being stimulated by the use of melibiose.

Nevertheless, it must be emphasized that the non-growing cultures used in these experiments allow many of the effects of melibiose to be observed, some or all of which may be related to enzyme induction, whereas in the exponentially growing cultures generally employed for induction experiments the effects of the inducer would probably be completely masked by the proportionately greater rates of metabolism of the cellular constituents. The final answer as to the gratuity of melibiose, and therefore as to the extent and specificity of its effects, must await further experiments.

The results of these induction experiments with non-growing cultures also extend the general observation that little or no lag time is observed before the onset of enzyme synthesis following addition of inducer. This is true even though the inducer is added after the cessation of culture growth, at a time when it might be expected that the bacteria would encounter difficulties in rapidly producing a complete enzyme-forming system. The observed immediate onset of synthesis at the maximal rate in these experiments supports the hypothesis of a stable, pre-formed, repressed enzyme-forming system which requires the presence of an inducer for its activation (50), and is consistent with the idea of stable templates on which proteins are synthesized. It has only recently been demonstrated that the actual site of enzyme formation in bacteria probably lies in the ribosomes (9), thereby implying that at least a portion of the

ribonucleic acid residing in these particles is a reasonably stable species. The great stability of the ribosomes to degradation by ribonuclease has been reported (32, 51).

Before it was possible to evaluate the nature of the ribonucleic acid turning over in the ribosomal and soluble fractions, and especially that portion that was dependent on the presence of the inducer, it was necessary to first obtain the base ratios of the bulk ribonucleic acid in each of the two fractions. These ratios then served as a reference against which the newly-formed ribonucleic acids were compared.

It is seen from the results that the base ratios of the induced and non-induced ribonucleic acid fractions are essentially the same. This correlation is as expected, since almost all of this material was formed prior to division of the primary culture into the smaller experimental cultures, and observable changes in concentrations of their constituent ribonucleic acids had long since ceased to occur. The result is that the bulk ribonucleic acid of the two cultures is identical except for whatever small changes occur during the non-growing experimental period. It is apparent that whatever changes do occur during this induction period, they are not sufficient to influence the overall base ratios of the bulk ribonucleic acid fractions present in the cell.

On the other hand, the base ratios of these bulk ribonucleic acid fractions as measured in exponentially growing cultures might very easily be significantly altered as changes

in ribonucleic acid metabolism occurred during the transition to the non-growing state. That this is the case is indicated by a comparison of the data obtained in these experiments with those obtained by Spahr and Tissieres (52) from ribosomal ribonucleic acid and by Dunn, Smith and Spahr (53) from soluble ribonucleic acid of exponentially growing Escherichia coli. The deviations in base ratios are not great, generally being between 1-2.5 per cent, but they appear to be significant. Of interest in this regard are studies by Santer, et al. (54), in which it has been shown that even in exponentially growing cultures the base ratios of the ribonucleic acid of Escherichia coli can be caused to vary as much as 3 per cent simply by changing from a glucose to a broth medium.

It was seen that the nucleotides isolated from the ribonucleic acid fractions of induced cultures had both a higher specific and total radioactivity than those isolated from the corresponding non-induced fractions. This clearly demonstrates that increased incorporation of label due to melibiose induction actually takes place in the ribonucleic acids themselves rather than in some undetected contaminant not removed in the phenol isolation procedure. However, the most interesting observation was related to the ratios of total activity found in each of the nucleotides. The results indicate that the distribution of the total activity among the nucleotides obtained by base hydrolysis of each of the ribonucleic acid fractions is the same for both the

induced and non-induced cultures. This is true even though considerably more radioactivity is incorporated into the induced fractions. It is possible that this similarity is accidental, even though the labeling proportions differ greatly from the distributions of the nucleotides themselves. The most likely interpretation of these data, however, is that melibiose stimulates the metabolic activity of specific fractions of ribonucleic acid typical of those already turning over in the non-growing culture. If this apparent stimulation is associated only with the beta-galactosidase synthesizing system, then it follows that the metabolism of ribonucleic acid associated with induction is probably closely related to that of the ribonucleic acids undergoing turnover in the non-induced culture.

Comparison of the overall nucleotide ratios of the bulk ribonucleic acid of the ribosomal and soluble fractions with the ratios of total radioactivity incorporated into each of the corresponding nucleotides of these fractions shows that the proportions are not identical. Closer scrutiny reveals that the ratios of total activity associated with the nucleotides differ from the bulk distributions of the nucleotides in what is apparently a very specific manner. In both the soluble and the ribosomal ribonucleic acid hydrolysates, it is in the labeling patterns of the purine nucleotides that large deviations from the bulk nucleotide ratios appear. In both fractions of the ribonucleic acid the deviation is in the direction of increasing the relative incorporation of P^{32}

into the adenine nucleotides while simultaneously decreasing the relative incorporation into the guanine nucleotides by approximately the same amount. The relative activities of each of the pyrimidine nucleotides are approximately the same as the proportion of the two nucleotides present in each of the two ribonucleic acid fractions.

This occurrence of consistent patterns of labeling among the induced and non-induced nucleotides, together with the fact that the distribution of total label among the nucleotides derived from each ribonucleic acid fraction differs widely from the overall nucleotide distribution of the corresponding ribonucleic acid fraction, is in itself subject to several interpretations. At first glance it might seem that the simplest explanation for these results would be that the free adenine nucleotide pool of ribonucleic acid precursors has a considerably higher specific activity than the guanine nucleotide pool within the non-growing bacterial cells. This explanation would suggest that the same precursor pools are used for the synthesis of both the soluble and ribosomal ribonucleic acids and that the nature of the ribonucleic acid molecules turning over under non-growing conditions, at least insofar as base ratios is concerned, is the same as that found in the bulk ribonucleic acid fractions formed largely during exponential growth of the culture.

This very reasonable-appearing hypothesis is quickly discredited, however, when a closer examination is made of

the procedures used to obtain the data. Of primary consideration is the general fact that alkaline hydrolysis of ribonucleic acid results in a cleavage of the ribose-phosphate bonds at each of the 5'-positions of the nucleotide sub-units composing the ribonucleic acid molecule. The liberated mononucleotides are in the cyclic 2', 3'-phosphodiester form, and under the continued influence of the alkali further hydrolysis occurs to yield the corresponding nucleoside-2'-and the nucleoside-3'-phosphates (55). It is these 2' and 3' isomers that were chromatographed on the anion exchange resin in the preceding experiments and which were analyzed for their total activities.

The preceding considerations must then be compared with what is known about the method of assembly of ribonucleic acid precursors into the final polynucleotide molecule. An enzyme has been isolated from E. coli by Littauer and Kornberg (56) that catalyzes the synthesis of high molecular weight polyribonucleotides from ribonucleotide-5'-diphosphates with the release of inorganic phosphate in a manner similar to that of the polynucleotide phosphorylase first discovered by Grunberg-Manago and Ochoa (57). More recent experiments, particularly those of S. B. Weiss (58, 59), have demonstrated the cell-free incorporation with the release of pyrophosphate of nucleoside-5'-triphosphates into a polymeric material that has all the characteristics of ribonucleic acid. The similarity of this system in every particular with the deoxyribonucleic acid-synthesizing system of Kornberg (60, 61)

gives strong presumptive evidence as to its importance in the synthesis of ribonucleic acid *in vivo*.

The important point from the point of view of this discussion is that the phosphate group carried into the ribonucleic acid molecule in each of the cited cases is originally attached to the 5'-position of the precursor. This phosphate is then transferred to the neighboring nucleotide at the 2' or 3' position during alkaline hydrolysis as described. Hence, any radioactivity found in a particular nucleotide following chromatography actually was incorporated into the ribonucleic acid as part of the nucleotide immediately adjacent to the one containing the label after alkaline hydrolysis.

In order to explain on the basis of variations in specific activities of ribonucleic acid precursor pools the relatively high incorporation of radioactivity found in the adenine nucleotides isolated after alkaline hydrolysis, variations must be anticipated specifically in the pools of those nucleotides that lie adjacent to the adenine in the ribonucleic acid chains. The absence of data concerning the mean nucleotide sequences of the ribonucleic acid of the ribosomal and soluble fractions makes it appear unlikely that a sequence of bases exists in both of the two fractions such that a specific combination of different specific activities of the precursors could be found that would yield the results observed. This is particularly true when the large variations in nucleotide content existing between the

two fractions of ribonucleic acid are considered. It appears that an explanation for the data must be sought on other grounds.

If the assumption were made that each type of nucleotide will be equally a neighbor to each of the four types of nucleotides present in ribonucleic acid, including its own, then it is readily apparent that the most extreme fluctuations in specific activities of the various precursor pools would have absolutely no effect on the relative amounts of radioactivity found in the four isolated nucleotides following base hydrolysis of the parent ribonucleic acid. In fact, the total amount of activity present in each of the nucleotides would be strictly proportional to the amount of that nucleotide present in the newly synthesized molecules if it is assumed that the entire molecule is synthesized de novo.

In the actual situation a base ratio of 1:1:1:1 for the four nucleotides is probably not present in the newly-formed ribonucleic acid and the probability would not be equal that a particular nucleotide would be the neighbor of each of the four types within the polymer. Acceptance of the identity of the base ratios of the newly-synthesized material with the corresponding ratios of total activity therefore requires the assumption of uniformly labeled precursor pools. The effects of small deviations from this ideal would probably cause small and unpredictable variations in the total activity ratios obtained.

With this assumption of uniformly-labeled pools of nucleotides, the ratios of total activity obtained in these experiments for the two fractions of ribonucleic acid represent the average base ratios of the small percentage of the total ribonucleic acid undergoing synthesis or turnover in these non-growing cultures. The facts that these ratios differ considerably from the base ratios of the bulk ribonucleic acid of the respective fractions, and that the ratios are not changed in the presence of the inducer melibiose even under the observed conditions of a 25-30 per cent stimulation in the rates of incorporation of labeled nucleotides in the induced cultures, offer strong support for the hypothesis of the presence of specific fractions of ribonucleic acid that have a relatively high metabolic activity under the conditions of the experiment.

There is one other possibility with regard to the total activity data that may be noted. It is perhaps possible that the small portion of the ribonucleic acid being metabolized is not strictly undergoing <u>de novo</u> synthesis, but rather represents the incorporation of fragments into pre-formed ribonucleic acid. It would be impossible to determine if these non-representative fragments were incorporated into the interior of the ribonucleic acid chains on the basis of these data or if the nucleotides were being added to the terminal positions of pre-formed molecules. It appears, however, that the data obtained for the soluble ribonucleic acid can not be explained on the basis of the known lability

of the three terminal nucleotides of the ribonucleic acid molecules involved in amino acid transfer present in the soluble fraction. Examination of the mode of linkage of these groups (62) shows that they are attached in the sequence cytidylic-cytidylic-adenylic to the carrier ribonucleic acid chain. The 5'-phosphate of the terminal adenylic acid is linked to the 3'-hydroxyl of the preceding cytidylic acid, and so on. Alkaline hydrolysis of this molecule would then yield adenosine, not adenosine phosphate. Two moles of cytosine phosphate would also be liberated, along with the general mixture of nucleotides from the bulk of the ribonucleic acid molecule. If the activity observed to be incorporated into the soluble RNA was derived primarily from the three terminal nucleotides, it would be expected that no P^{32} would be found in the adenylic acid and two moles of P^{32} would be found in the cytidylic acid for every three moles of radioactive phosphate incorporated. The distribution of activity would, of course, be modified by the transfer of the third mole of P^{32} into the nucleotide adjacent to the second labile cytidylic nucleotide. The hypothetical pattern of labeling just described is greatly different from that actually obtained. It can be seen that even if the small fraction of ribonucleic acid being metabolized in these non-growing cultures is associated with specific regions of pre-formed molecules, the total activity ratios could still represent the base ratios of that portion of the ribonucleic acid being metabolized. This interpretation

would have to be based on the earlier assumption of uniformly-labeled precursor pools plus the additional assumption that the newly-formed regions of the RNA molecules are long enough to make negligible the effects of shifting during alkaline hydrolysis a labeled phosphate to the adjacent nucleotide of the stable and unlabeled ribonucleic acid chain. Their lengths must also be sufficiently great to render negligible the effects of forming nucleotides and/or atypical nucleotides from the terminal nucleotides of the ribonucleic acid molecule. In fact, one possible explanation of the observed data might be just such statistical variations resulting from having rather short sequences of labeled nucleotides which terminate in a guanine nucleotide at the distal end and which are attached to "acceptor" adenine nucleotides at the 3' terminal position of the stable ribonucleic acid molecules. During hydrolysis the guanine nucleotides would selectively lose label and the adenine nucleotides would selectively gain. However, the probability of such a specific mechanism being applicable to the turnover observed in both the soluble and ribosomal ribonucleic acid fractions seems remote.

Chitosan chromatography of the ribosomal and soluble ribonucleic acids indicates that each may be composed of several distinct sub-fractions. The ribosomal ribonucleic acid is seen to divide into two major and at least three minor sub-fractions under the conditions employed in these experiments and the soluble ribonucleic acid divides into

two major and at least two minor sub-fractions. Although it is probable that each of these sub-fractions contains a heterogeneous population of molecules, the demonstration that the peaks rechromatographed at precisely the same point in the elution sequence as that at which they were originally eluted supports the contention that the observed grouping results from some fundamental similarity among the molecules constituting any one peak. Whether this similarity is one of molecule size, composition, structure or some combination of these or other parameters is not answerable on the basis of the observed data.

There is some indication that the divisions are at least partially related to metabolic, and therefore presumably functional, dissimilarities in the molecules. It seems reasonable that ribonucleic acid molecules having different functions in the bacterial cells would have different rates of incorporation of the P^{32}-labeled precursors in the non-growing cultures. It would also be expected under these circumstances that any change in the metabolism of specific fractions of ribonucleic acid caused by the presence of the inducer melibiose would be detected by variations in the relative rates of incorporation of P^{32} into corresponding ribonucleic acid fractions isolated from induced and non-induced cultures. It is seen that the fractions of ribonucleic acid obtained by chitosan chromatography differ from each other in their specific and total activities, and in their response to the inducer melibiose. Since there were

no observable differences between the quantities of the ribonucleic acid contained in the corresponding induced and non-induced sub-fractions, the ratios of the specific activities of these fractions are equivalent to the ratios of their total activities. These ratios may then be used as a measure of the degree of stimulation of the incorporation of labeled nucleotides into the ribonucleic acid of the induced compared to the non-induced chromatographic fractions. An analysis of the P^{32}-content of the fractions of ribosomal ribonucleic acid obtained after column chromatography indicates that that fraction remaining adsorbed on the exchanger after completion of the elution sequence turns over in the non-growing bacteria at a more rapid rate than any of the eluted fractions. Induction with melibiose apparently causes almost no change in the rate of incorporation of label into the adsorbed fraction, whereas there is a very pronounced stimulatory effect on the material in the largest of the eluted fractions. Among the minor ribosomal fractions, there is none whose rate of metabolism shows a significant tendency toward being stimulated by the presence of melibiose, although each becomes labeled in the non-growing culture. It can be concluded that of the various fractions of ribosomal ribonucleic acid separable by chitosan chromatography, one in particular exhibits a greatly increased rate of metabolism under the influence of the inducer melibiose.

Analysis of the chromatographic fractions of the soluble ribonucleic acid shows a much more complex pattern of labeling

than that observed for the ribosomal fractions. The incorporation of P^{32} into the first small fraction to be eluted from the column proceeds relatively slowly in non-induced cultures. Nevertheless, the ratio of the amount of isotopic phosphorus that is incorporated into this fraction in the induced as compared with the non-induced cultures exceeds that for any of the other soluble fractions. The stimulation of incorporation of labeled phosphate into both the second fraction, which contains the major portion of the soluble ribonucleic acid, and the last minor fraction to be eluted, occurs to an intermediate extent when the cultures are grown in the presence of melibiose. The metabolism of the remaining large fraction does not seem to be greatly influenced by the inducer.

It appears on the basis of the preceding experiments that the observed ability of melibiose to stimulate the incorporation of P^{32}-phosphate into various cell fractions of non-growing Escherichia coli is actually quite specific in nature. The stimulation apparently does not apply to the deoxyribonucleic acid, to large fractions of the ribosomal ribonucleic acid, or to a large fraction of the soluble ribonucleic acid of the bacterial cells. However, a considerable increase was observed in P^{32} incorporation in the lipids extracted from both the ribosomal and the soluble fractions of the induced cultures when these were compared with those obtained from the corresponding

fractions of the non-induced cultures. Similarly, the rates of incorporation of P^{32} into certain specific chromatographic fractions of both the ribosomal and soluble ribonucleic acid increased when the non-growing cultures were exposed to the inducer. It would be interesting to determine if the lipid fractions could be similarly fractionated with respect to the effects of melibiose on the stimulation of P^{32}-incorporation.

If it is assumed that all the effects of melibiose observed in these experiments are directly related to the induction of beta-galactosidase, then several conclusions concerning the induction process may be drawn.

First, the induction process apparently does not require a change in the metabolism of cellular deoxyribonucleic acid. If deoxyribonucleic acid is required in the system, the material already present in the cell prior to induction seems to be capable of performing the necessary functions.

Second, the induction process apparently results in an increased synthesis or turnover of the phospholipids of both the ribosomal and soluble ultracentrifugal fractions obtained in these experiments. These lipids are possibly associated with protein in the native state.

Third, the induction process apparently results in the increased metabolism of both the soluble and ribosomal ribonucleic acid fractions of the bacteria as measured by increases in P^{32}-phosphate incorporation.

Fourth, the base ratios of the ribonucleic acid undergoing increased synthesis or turnover during induction are different from the base ratios of the bulk ribonucleic acid of the parent fractions. The per cent compositions of these rapidly-metabolizing fractions have been calculated on the basis of total P^{32}-incorporation data. The data also indicate that the nucleic acids synthesized as a result of induction have the same base compositions as those turning over in the non-growing non-induced cultures. It also appears that in both rapidly metabolizing fractions the proportion of adenine is increased and that of guanine decreased when compared with the bulk ribonucleic acid of that fraction.

Fifth, induction appears to stimulate incorporation of P^{32}-phosphate disproportionately among the various sub-fractions of ribonucleic acid obtained by chitosan chromatography of both the ribosomal and soluble ribonucleic acid. In each case this increased incorporation appears to be localized primarily in one major sub-fraction.

On the other hand, it is possible that any or all of these effects of melibiose are due to a selective stimulation by the compound of the metabolism of the fraction concerned rather than being related directly to the induction phenomenon. If this should be the case, it is still tempting to postulate that the observed effects may nevertheless be specifically related to the processes of protein biosynthesis. Such an hypothesis is based on the reasonable possibility that the ability of melibiose to relieve the repression of beta-

galactosidase synthesis may be only an indication of its capacity to stimulate the general synthesis or turnover of proteins in the cultures studied. The turnover of the phospholipids may then reflect the turnover of the lipoproteins of which they are a part. The results of the experiments on the possible inhibitory effects of 5-hydroxyuridine may offer some means of choosing between the two possibilities.

As discussed in the introduction to this dissertation, the inhibitory effects of base analogs of ribonucleic acid precursors on protein synthesis forms one of the primary supports for the hypothesis that protein formations depends on ribonucleic acid synthesis.

In one hypothesis, it is assumed that the inhibition by base analogs is caused by an alteration of the structure of a specific species of ribonucleic acid that is then unable to perform properly its function in the synthesis of an enzyme. It does not seem likely that the relatively stable ribosomal ribonucleic acid would be affected quickly enough in this manner to explain the action of the inhibitors in halting enzyme formation immediately after being added to the culture. Chantrenne suggests the more likely alternative that the base analogs are most rapidly incorporated into a ribonucleic acid fraction capable of very active renewal, such as the soluble ribonucleic acid, and that the rapid formation of these abnormal molecules leads to the sudden decrease in protein synthesis (63).

In the present experiments it was seen that 5-hydroxyuridine was totally unable to influence the rate of formation of beta-galactosidase in non-growing cultures. This result was obtained even when a concentration of the inhibitor was used which was one hundred times that normally required for total inhibition of beta-galactosidase synthesis in growing cultures. If incorporation of the analog into sensitive ribonucleic acid fractions is accepted as the reason for the ability of the inhibitor to cause cessation of beta-galactosidase synthesis, it is apparent that insufficient turnover of this sensitive ribonucleic acid species occurs in the non-growing bacteria to allow inhibition to occur. It seems likely that in rapidly dividing bacteria the new analog-containing ribonucleic acid might quickly interfere with the function of the normal ribonucleic acid. In non-growing bacteria the rate of synthesis of the ribonucleic acid species required for enzyme formation is probably so low as to prevent inhibition by the analog-modified species. Should such be the case, it is a logical conclusion that the ribonucleic acid species required for the synthesis of beta-galactosidase are pre-formed and capable of being activated to the maximum extent by an inducer even without the further production of normal ribonucleic acid.

An alternative possibility often presented is that ribonucleic acid synthesis must occur simultaneously with enzyme synthesis. Incorporation of the analog into the nucleic acid under these conditions then might be expected to result in

the disturbance of the normal processes of enzyme formation. Since the presence of large quantities of such an analog in these non-growing cultures had no effect on formation of the enzyme, it follows that the synthesis of protein did not require the simultaneous synthesis of ribonucleic acid, if it is assumed that synthesis of normal nucleic acid could not occur in the presence of the analog.

In conclusion, it must be admitted that the original goal of determining the effects of induction on ribonucleic metabolism have not been completely achieved. The data obtained in these experiments are not sufficient to establish a direct correlation between the observed ribonucleic acid turnover and synthesis of the induced enzyme, and the present state of knowledge in this area does not allow a decision to be made as to the degree to which the induction process alone was responsible for the observed results. There were several significant observations made in the course of these experiments which appear to relate directly to the problem of ribonucleic acid metabolism and induction in non-growing Escherichia coli.

A slow generalized turnover in the absence of growth was observed for every sub-cellular molecular species examined in the resting cultures of Escherichia coli. This dynamic state provides a minimum metabolic background against which any radioactive incorporation experiments may be expected to be viewed. Addition of melibiose to the culture medium increases incorporation of P^{32}-phosphate into these

species over the very low background levels to a variable extent, ranging from none at all in the deoxyribonucleic acids and some chromatographic fractions of cellular ribonucleic acids, to 25-30 per cent in the lipids and some of the more active ribonucleic acid chromatographic fractions. Variations appear to occur to the greatest extent among the several species of ribonucleic acid obtained by chromatographic separation.

It appears from these experiments that the P^{32} that is incorporated into both the ribosomal and soluble ribonucleic acid fractions as a result of melibiose induction is incorporated selectively in molecular species having base ratios different from the base ratios of the corresponding parent fraction. The newly synthesized molecules appear in both fractions to be high in adenine and low in guanine. Of interest is the fact that the nucleic acid formed or turned over in response to melibiose stimulation appeared to be similar to that already turning over in the non-induced non-growing bacteria.

Finally, the complete lack of inhibition by 5-hydroxyuridine of the induced formation of beta-galactosidase indicates that enzyme synthesis is not dependent upon ribonucleic acid synthesis, and that all the nucleic acid species necessary for the synthesis of inducible enzymes are present in significant amounts prior to the addition of the inducer. The results also indicate that activation of the inducer, if it is necessary, does not require the de novo synthesis of ribonucleic acid co-factors.

SUMMARY

SUMMARY

Melibiose induction of beta-galactosidase in non-growing cultures of Escherichia coli has been shown to exert a general stimulatory effect on the metabolism of several compounds obtained from the soluble and ribosomal ultracentrifugal fractions of the bacterial extracts. Deoxyribonucleic acid and some sub-fractions of ribonucleic acid are exempt from this stimulation, whereas lipids and some sub-fractions of ribonucleic acid undergo quite pronounced stimulation as measured by P^{32}-phosphate incorporation into the fractions.

The species of ribonucleic acid turning over during induction were analyzed for their base ratios and were found to differ in this respect from the bulk ribonucleic acid of which they were a part. Comparison of the ribonucleic acid turning over during induction with that turning over in the absence of inducer in the non-growing bacteria shows that they are of apparently the same base composition. Chromatographic fractionation of the ribonucleic acid fractions demonstrated within them the presence of specific sub-fractions which responded in varying degrees to the presence of the inducer.

The failure of the uridine analog 5-hydroxyuridine to inhibit the synthesis of beta-galactosidase in the non-growing

cultures was taken as evidence for the dissociation of enzyme formation and ribonucleic acid synthesis in that instance.

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APPENDICES

APPENDIX I

MAINTENANCE AND CULTURING OF ESCHERICHIA COLI ML 30

Materials:

1. Defined salts media:

   A. Defined salts medium of Monod, et al.: (27)

Potassium dihydrogen phosphate	13.6 g.
Ammonium sulfate	2.0 g.
Magnesium sulfate heptahydrate	0.2 g.
Calcium chloride	0.01 g.
Ferrious sulfate heptahydrate	0.0005 g.
Distilled water	to 2 l.
Adjust pH with potassium hydroxide solution	to pH 7.4

   B. Defined salts medium of Volkin and Astrachan (28):

Ammonium chloride	2.0 g.
Ammonium sulfate	2.0 g.
Sodium chloride	20.0 g.
Potassium chloride	2.0 g.
Magnesium chloride hexahydrate	0.81 g.
Calcium chloride dihydrate	0.59 g.
Sodium monohydrogen phosphate dodecylhydrate	0.5 g.
Tris	25.0 g.
Distilled water	to 4.0 l.
Adjust pH with 1 N hydrochloric acid	to pH 8.0

2. Culture medium: To two liters of the desired defined salts medium are added 6.0 g. of glucose in a 5 liter Erlenmeyer flask. The flask is plugged with cotton and minimally autoclaved at 15 p.s.i. for 12 minutes. Extended periods of autoclaving were found to cause decomposition of the glucose. The flask is placed in an ice bath as soon as possible after being removed from the autoclave.

3. Culture slants: The bacteria are maintained on culture slants consisting of culture medium containing 1.5 per cent bacto-agar. The agar is dissolved in culture medium by heating (caution: foams) and 10 ml. fractions of the resulting solution are

transferred to Pyrex tubes while still hot. The tubes are stoppered with cotton plugs and sterilized by minimally autoclaving. The tubes are placed on a slant while still hot to provide maximum surface area and the contents are allowed to gel. The hardened slants are incubated at 37° C. for 24 hours to detect accidental contamination. They are then stored at 10° C.

4. Inoculating tubes: Ten milliliter portions of culture medium are placed in Pyrex tubes which are of such a size as to permit their use in a Klett-Summerson photoelectric colorimeter. The tubes are plugged with cotton, minimally autoclaved as described, and stored at 10° C. after being incubated to detect contamination.

Procedure:

1. A loopful of bacteria is transferred from a slant to an inoculum tube at monthly intervals. The inoculum culture is grown with gentle shaking at 37° C. until turbid with bacteria, after which a loopful of the inoculum is used to inoculate a new slant in zig-zag fashion, from bottom to top, with rapidly growing bacteria. The newly-inoculated slant is incubated at 37° C. for 24 hours to allow a heavy growth of bacteria to develop on its surface. It is then stored at 10°C. for further use.

2. Inocula: When used for inoculating a large culture, the inoculum turbidity is measured periodically and the desired volume is transferred by means of a sterile pipet to the culture flask. Accurate timing of the culture growth requires the use of as large and turbid an inoculum as possible.

3. Growth of Culture: All bacterial cultures and inocula used in these experiments were grown in a small constant temperature room of home-made construction. Aerobic growth of the cultures was insured by shaking them on a variable speed platform shaker. The speed was varied to meet the requirements of the culture and flask size for any particular experiment to yield maximum shaking without splashing.

APPENDIX II

CULTURE ASSAY METHODS

Treatment of Culture with Toluene for Stock Assay Solution

The bacteria in a culture of Escherichia coli are caused to undergo lysis in a reproducible manner by saturating the medium with toluene according to the method of Novick and Wiener (29).

Reagents: 1. Toluene

2. Sodium deoxycholate solution containing 1 µg./ml. (optional). Dissolve 1 mg. sodium deoxycholate in 1 liter of water.

Procedure: 1. Immediately after determining the culture turbidity of the bacterial culture pipet 5.0 ml. of the culture into a 10 inch test tube containing 0.1 ml. of toluene and (optional) 0.05 ml. of sodium deoxycholate solution.

2. Stopper the tube, place on an angle in a test tube rack and shake at 30° C. for 10 minutes.

3. The resulting stock assay solution may be safely stored for one week at 10°C. since the protein and beta-galactosidase assay were found to be independent of this storage time. It should be thoroughly shaken before use with a short period being allowed thereafter for settling of the toluene layer.

Culture Protein Determination

Total protein in the bacterial cultures is determined by a slight modification of the method of Lowry, et al. (30).

This is a colorimetric method based on the production of a blue color by the action of copper tartrate on certain chromagenic groupings in proteins in conjunction with reduction of the Folin-Ciocalteu phenol reagent in alkaline solution (40).

Reagents:

1. Reagent A: 2% sodium carbonate in 1.0 N sodium hydroxide. Dissolve 4 g. of sodium hydroxide and 20 g. of sodium carbonate in sufficient water to make 1 liter.

2. Reagent B: 0.5% cupric sulfate pentahydrate in 1% sodium or potassium tartrate. Dissolve 0.5 g. of cupric sulfate pentahydrate plus 1 g. of potassium tartrate in sufficient water to make 100 ml.

3. Reagent C: Alkaline Copper Solution. Mix 50 ml. of Reagent A with 1 ml. of Reagent B. Discard after 1 day.

4. Reagent E: Folin Reagent. Purchased as Folin-Ciocalteu phenol Reagent from the Hartman-Ledden Company and used full strength.

Procedure:

1. Pipet 0.5 ml. of stock assay solution prepared as above into a 10 inch test tube. A distilled water blank is carried simultaneously through the same procedures.

2. Add 5.0 ml. of Reagent C to the tube. Mix well and let stand at least 10 minutes at room temperature. Then add by means of a blow-out pipet 0.5 ml. of Reagent E and mix well. The time required for this addition and mixing should not exceed 2 seconds. After standing a minimum of 30 minutes more read at 750 mμ in the Beckman DU.

3. The absorbancy reading is corrected for values obtained from the reagent blank and from a turbidity blank obtained by diluting 0.5 ml. of the stock assay solution with 5.5 ml. of distilled water. A standard of bovine serum albumin may be made up and carried through the procedure if it is desired to obtain a somewhat quantitative estimate of the actual rather than relative amounts of protein in the stock solutions. This is not necessary for purposes of plotting a growth curve, however.

Culture RNA Determination

The concentration of the ribonucleic acid in the bacterial cultures was estimated by the method of Dische and Schwarz which is described in detail in Appendix III.

Procedure: 1. To 1 ml. of the stock assay solution is added 0.5 ml. of water and this diluted sample is analyzed for ribonucleic acid.

2. Since unused glucose in the medium will interfere with this determination, the growth curve plotted from these data are valid only in the plateau, or non-growing region, at which time the glucose has been exhausted. The concentration of the melibiose on the other hand remains constant at all times, and may be corrected for.

Culture beta-Galactosidase Determination

The concentration of beta-galactosidase in the bacterial cultures was determined by a modification of the method used by Rickenberg and Lester (26). This is a colorimetric enzyme assay based on the formation of the yellow chromogen o-nitrophenol when the artificial substrate o-nitrophenyl-beta-D-galactoside (ONPG) is hydrolyzed by the enzyme beta-galactosidase.

Reagents: 1. ONPG Reagent: 0.1 M sodium phosphate buffer, pH 7.0, made 0.0025 M in ONPG (obtained from California Corporation for Biochemical Research; MW301.2). Dissolve 1.38 g. of sodium dihydrogen phosphate monohydrate and 0.0753 g. of ONPG in approximately 75 ml. of water. Adjust to pH 7.0 and make up to 100 ml. with water. This reagent is stable for months when stored at 10°C.

2. 1 M potassium carbonate solution, made by dissolving 138.2 g. in 1 liter of water. This reagent may be stored indefinitely in a well-stoppered bottle.

Procedure: 1. 0.2 ml. of the stock assay solution obtained from the toluene-treated culture are pipetted into a Klett colorimeter tube containing 3.0 ml. of the ONPG reagent at 37°C. A water blank is also carried through the entire procedure.

2. After exactly 20 minutes, 2.0 ml. of the 1 M potassium carbonate solution are added to stop the reaction and develop the color of the o-nitrophenol.

3. After 5 minutes more the color developed in the solutions is read in a Klett-Summerson photoelectric colorimeter at 420 mμ. After correction for the values of the blank the colorimetric readings give values which are proportional to the amount of enzyme present in the stock assay solution.

APPENDIX III

GENERAL ASSAY METHODS

Deoxyribonucleic Acid Determination

The colorimetric method of Dische (41) was used to determine deoxyribonucleic acid.

Reagents:

1. The combined digestion and color reagent is made up by dissolving 0.41 g. of diphenylamine, twice recrystallized from 70% ethanol, in 38 ml. of glacial acetic acid and adding 1 ml. of concentrated sulfuric acid.

Procedure:

1. One volume of a solution of the unknown is added to two volumes of the Dische reagent in a pyrex test tube. The tube is placed in a boiling water bath for 30 minutes. A standard solution of deoxyribonucleic acid and a water blank are run simultaneously.

2. The color intensities developed in the tubes are readin the Beckman DU at 595 mμ. The value obtained for the blank is subtracted from the sample reading.

Deoxyribonucleic Acid Determination

The colorimetric method of Stumpf (42) was used as an auxiliary means of determining deoxyribonucleic acid.

Reagents:

1. A 5% solution of cysteine hydrochloride is made by dissolving 1.25 g. of cysteine hydrochloride in 25 ml. of water.

2. A 70% solution of sulfuric acid, made by adding 38.1 ml. of concentrated sulfuric acid to 30 ml. of water.

Procedure:

1. Thoroughly mix in a test tube 0.05 ml. of 5% cysteine hydrochloride solution, 0.5 ml. of the solution containing DNA, and 5 ml. of sulfuric acid solution. A standard solu-

tion of DNA containing 0.5 mg. DNA per ml. may be carried through the procedure if desired.

2. After standing at room temperature for 10 minutes the color is read in the Beckman DU at 490 mμ.

Ribonucleic Acid Determination

The orcinol color test according to Dische and Schwarz (43) was used for the determination of ribonucleic acid.

Reagents: 1. Digestion and color reagent: Dissolve 100 mg. of ferric chloride hexahydrate in 6 N hydrochloric acid. Add 3.5 ml. of a 6% solution of orcinol (twice recrystallized from benzene) in ethanol.

Procedure: 1. To 1.5 ml. of a solution of ribonucleic acid is added 3 ml. of the digestion and color reagent. A water blank and a standard should be run concurrently. Mix and heat in a boiling water bath for 3 minutes, then cool in tap water.

2. Measure the absorbancy of the sample in the Beckman DU at 665 mμ. The value obtained for the water blank should be subtracted from the sample and the difference obtained may be compared with the standard. The absorbancy is proportional to the concentration of the ribonucleic acid in the range between 10 and 100 μg. per ml.

Protein Determination

The biuret reaction according to Robinson and Hogden was used for the estimation of protein (44).

Reagents: 1. Biuret reagent: Dissolve 1.5 g. of cupric sulfate pentahydrate and 6.0 g. of sodium potassium tartrate tetrahydrate (Rochelle salt) in approximately 500 ml. of water in a liter volumetric flask. Add to this solution with constant swirling 300 ml. of freshly-prepared carbonate-free 10%

sodium hydroxide. Make up to 1 liter with water and store in polyethylene bottles. Discard if a black or red precipitate appears.

Procedure: 1. A solution containing 1 to 6 mg. of protein is placed in a Klett tube. An equal volume of the Biuret reagent is added and mixed. After standing 30 minutes the color is measured in the Klett-Summerson photoelectric colorimeter using the 540 mμ (green) filter. A water blank should also be carried through the procedure. Its color intensity is subtracted from that of the sample as a correction for the reagents.

Organic and Total Phosphorus Determination

Total phosphorus was determined by a modification of the method of King (45). This is a colorimetric method based on the production of a blue color by the reaction of acid phosphomolybdate with a 1-amino-2-naphthol-4-sulfonic acid(ANSA)-bisulfite reducing reagent.

Reagents: 1. Digestion reagent: 10 N sulfuric acid.

2. Stable reducing mixture: Mix 29 g. of sodium bisulfite, 1 g. of sodium sulfite and 0.5 g. of ANSA in a mortar. Grind to a fine powder with a pestle and store in a brown bottle in the cold.

3. Reducing reagent: Dissolve 3 g. of the stable reducing mixture in 20 ml. of water at room temperature just before use.

4. Acid molybdate solution: 272 ml. of concentrated sulfuric acid are added to 700 ml. of water with cooling. Add with mixing 50 g. of ammonium molybdate in 1 liter of water, and adjust total volume to 2 liters.

5. Phosphate standard: 0.136 g. of potassium dihydrogen phosphate (Merck) are dissolved in 1% trichloroacetic acid to exactly 1 liter. The standard solution contains 1 μmole of phosphorous per ml.

Procedure:

1. Samples containing an unknown amount of phosphorous are digested on a digestion rack with 0.4 ml. of 10 N sulfuric acid in a pyrex tube containing one or two glass beads to prevent bumping.

2. After 30-60 minutes the tubes are removed, partially cooled, and 1-2 drops of 30% hydrogen peroxide are added. Heat the tubes for 15-20 minutes more, partially cool, and add 1 ml. of water to the colorless residue.

3. Heat the diluted sample in a boiling water bath for 10 minutes to hydrolyze pyrophosphate. This solution is used in the colorimetric assay for inorganic phosphate which follows.

4. Make the samples up to 5 ml. with water. Also prepare a water blank and a standard to be carried through the procedure. Add 1 ml. of molybdate reagent followed by 1 ml. of reducing reagent. Add 3 ml. more of water and mix well.

5. The solutions are allowed to stand for 20 minutes and then are read in a Klett-Summerson photoelectric colorimeter with the 640 mμ (red) filter. The amount of phosphorus in the unknown is calculated by comparison of the readings with that of the standard after correcting for the blank.

6. To determine organic phosphorous, determine the inorganic phosphorous content of the material by carrying the samples through steps 4 and 5 of this procedure. Subtract the value obtained for inorganic phosphate content of the samples from those obtained as total phosphate in the procedure above.

APPENDIX IV

PREPARATIVE METHODS FOR NUCLEIC ACIDS

Deoxyribonucleic Acid

Undegraded deoxyribonucleic acid was isolated by the method of Sevag (33).

Reagents: 1. Chloroform-isoamyl alcohol deproteinization reagent: Mix 75 ml. of chloroform with 25 ml. of isoamyl alcohol yielding a 3:1 (v/v) solution.

Procedure: 1. To the solution containing deoxyribonucleic acid is added in a centrifuge tube sufficient solid sodium chloride to make a 10% solution.

2. Add 1/2 volume of the deproteinizing reagent, cap the tube, and shake vigorously for 10 minutes. Following the shaking, centrifuge the mixture at 10,000Xg. for 10 minutes. The aqueous layer containing the deoxyribonucleic acid is removed by pipet and transferred to another centrifuge tube. More of the deproteinizing reagent is added and the procedure is repeated indefinitely, generally around nine or ten times, until no further protein appears at the organic-aqueous interface.

Ribonucleic Acid

Undegraded ribonucleic acid was isolated by a modification of the phenol procedures of Gierer and Schramm (35) used for TMV ribonucleic acid, and of Kirby (36), used for preparation of mammalian ribonucleic acid.

Reagents: 1. Water-saturated phenol: 300 ml. of aqueous chromatographic grade 88% phenol, obtained from Mallinckrodt without added preservative, is thoroughly mixed with 75 ml. of water and equilibrated at 4°C. The lower layer is used in the preparative procedures which follow.

Procedure: 1. The solution of ribonucleic acid is placed in a separatory funnel and shaken for 8 minutes with an equal volume of water-saturated phenol. The emulsion is centrifuged for 10 minutes at 5000 x g in a refrigerated centrifuge and the aqueous (upper) layer containing the RNA is removed by pipet. The aqueous layer is extracted twice more with 1/2 volume of aqueous phenol, followed each time by centrifugation and pipetting of the aqueous layer. If desired, the phenol layers may be pooled and back-extracted with a small amount of water which is combined with the original aqueous layer to give higher yields of RNA.

2. The aqueous layer is extracted three times with equal volumes of ethyl ether to remove the phenol. During the initial ether extraction a jelly-like emulsion is generally present at the ether-water interface. This material was found to persist in the subsequent extractions unless it was discarded as part of the ether layer when the layers were separated and removed from the separatory funnel. Some residual material, assumed to be a protein emulsion, was also rinsed from the sides of the separatory funnel and discarded before continuing with the ether extractions to insure its complete removal from the aqueous layer. The material is not present after subsequent extractions if these precautions are taken.

3. The aqueous solution contains only RNA and polysaccharide. It characteristically gives a positive orcinol and negative Dische, Stumpf and biuret tests (see Appendix III).

APPENDIX V

SPECTROPHOTOMETRIC CONSTANTS OF NUCLEOTIDES

Spectrophotometric Constants of Nucleotide 2' and 3' Phosphates at pH 2.0 abstracted from the data of Volkin and Cohn (39).

Compound	$\epsilon_{260} \times 10^{-3}$	$\epsilon_{250}/\epsilon_{260}$	$\epsilon_{280}/\epsilon_{260}$	$\epsilon_{290}/\epsilon_{260}$
Cytidine phosphate	6.8	0.465	1.90	1.325
Adenosine phosphate	14.2	0.85	0.22	0.03
Uridine Phosphate	9.9	0.78	0.30	0.03
Guanosine phosphate	11.8	*1.02	0.68	0.40

*The pH is critical for the accuracy of this value. The others are generally valid throughout the acid pH range.