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INTRODUCTION

Subject and Objectives

The general objectives of this project were set forth in the last six month report and will not be reiterated. New topics to be investigated include the subdivision and reconstitution of ribosomes, evolution of certain enzyme systems in Pseudomonas, and studies of the molecular mechanism in the assembly of bacteriophage T4 from its protein units to form an infective organism.

5'-TERMINAL NUCLEOTIDE SEQUENCES OF RIBOSOMAL RNA

(M. Takanami, S. Goolsby, J. Levitt)

In the last six month report, the 5'-terminal 23S RNA and 16S RNA obtained from the ribosomes in E. coli were identified as pGpPupPyp and pApApApypGp respectively. Further research has shown the fragments are pGpGpU and pApApApUpG. These are the first sequences to have been identified in any ribosomal RNA. Work is continuing on identifying the beginning sequences of ribosomal RNA from other organisms.

FUNCTION AND STRUCTURE OF BACTERIAL RIBOSOMES

(K. Hosokawa, S. Goolsby)

The reconstitution in vitro of functionally active ribosomes of E. coli (Hosokawa, et al., 1965; Staehelin, et al., 1966) led to a new way of elucidating relationships between function and structure of ribosomes.

When ribosomes (30S or 50S, p = 1.6) were subjected to CsCl equilibrium density gradient centrifugation, a specific protein fraction was set free ("split proteins"), while the residue of ribonucleoprotein particles formed a band of density 1.65, called "core particles" (23S or 40S). By mixing the split proteins with core particles, units

with the original sedimentation constant are formed which have the original function of the intact ribosomes as indicated by polyuridylic acid-directed phenylalanine incorporation.

If the split proteins could be further fractionated into subcomponents and recombined with core particles in a systematic way, we might expect that the functions of ribosomes could be divided into the attachment of messenger RNA, the attachment of amino-acyl transfer RNA, and the formation of peptide chains.

For fractionation of split proteins, a method of preparing pure ribosomal proteins in large quantities is necessary. This was developed by improving the method of cell extraction and by using zonal sucrose gradient centrifugation. Fractionation of split proteins of 30S ribosomes was carried out by DEAE-cellulose column chromatography in the presence of 6-molar urea. The basic protein fraction which passed through the first column was further fractionated into each ultimate subcomponent by cellulose cation exchanger column chromatography. Experiments to determine the respective functions of these protein components and characterization of these subcomponents are now in progress. (Part of this work is being carried out in collaboration with Dr. Nomura's group at the University of Wisconsin, and Dr. Hiroshi Matsubara.)

Future Plans

1. Ribosomes of thermophilic and psychrophilic organisms will be examined to explore the influence of temperature on a living system and the acquisition of resistance to temperature, using the technique of reconstitution of hybrid ribosomes. E. coli will be used as a control.

2. Agents such as antibiotics and mutational modifications of ribosomal structure, which cause misreading of codons, will be used to investigate the fine structure of ribosomes.

EVOLUTION OF HYDROXYLASES OF AROMATIC COMPOUNDS IN PSEUDOMONAS

(K. Hosokawa, J. Levitt, S. Goolsby)

Pseudomonads, soil bacteria, are capable of growing on various unusual organic compounds. The agents which primarily attack these compounds are inducible enzymes.

Pseudomonas putida M-6 (ATCC No. 17428), which was isolated from soil (K. Hosokawa, 1959) shows a wide growth spectrum; for example, it grows on p-hydroxybenzoic, salicylic, benzoic or anthranilic acids as sole carbon and energy sources. The close similarities of the enzymes in analogous reaction steps in these metabolic pathways indicate that they were differentiated from a single enzyme prototype or a single genome as a result of gene duplication and modification during evolution. A comparison of p-hydroxybenzoate and salicylate hydroxylases will be undertaken to investigate this. The former is the primary enzyme that attacks p-hydroxybenzoate and was obtained crystalline (K. Hosokawa, et al., 1966). It contains FAD as a prosthetic group and requires NADPH and molecular oxygen as substrate besides p-hydroxybenzoic acid (K. Hosokawa, et al., 1966). Salicylate hydroxylase is formed inducibly and is known to be very similar to the former except for a requirement of NADH rather than NADPH. P-hydroxybenzoate hydroxylase is split into at least four subunits of different electrophoretic mobility (K. Hosokawa, unpublished results). It is very likely that salicylate hydroxylase also consists of subcomponents. If two hydroxylases have the same evolutionary origin, they might have common subunits. This could be demonstrated if a hybrid enzyme could be reconstituted from subcomponents of both enzymes. To this end purification of the two hydroxylases is under progress.

Future Plans

1. Comparative biochemistry of hydroxylases (mainly protein chemistry).
2. Evolutionary aspects of genetic control mechanisms of hydroxylases.

STUDIES OF DNA REPLICATION IN BACILLUS SUBTILIS

(H. Yoshikawa, M. Haas, H. Mayoh, B. Jansen, E. Cook)

DNA Synthesis During and After Thymineless Germination of Bacillus Subtilis Spores

Spores of a thymine-requiring mutant of B. subtilis (leu^-, trp^-, thy^-) were germinated in a synthetic medium lacking thymine. DNA synthesis started immediately after the addition of thymidine (TdR) or 5-bromodeoxyuridine (5BUdR) following a thymine starvation period of 3 hours. Sequential replication of genetic markers was also observed during the DNA synthesis with 5BUdR.

This system provided an opportunity of studying a model of DNA replication immediately after the initiation of chromosome replication. For this purpose the density of DNA labelled with ^{3}H-BUdR for 30 seconds and 1 minute was determined by CsCl density gradient centrifugation with and without alkali treatment and hydrodynamic shearing.

The results were as follows:

1. Without shearing the densities of both native and alkali-denatured DNA labeled with ^{3}H-BUdR were only slightly higher than that of a normal light DNA. These densities were independent of the concentration of 5BUdR.

2. Shearing at 80 V for 10 minutes produced a hybrid DNA from the native ^{3}H-BUdR labelled DNA and a denatured heavy DNA from alkali-denatured ^{3}H-BUdR labelled DNA.

3. Approximately 50% of the ^{3}H-BUdR DNA was chased into a hybrid DNA by the incubation with non-radioactive 5BUdR.

These results ruled out the possibility that the lower density of ^{3}H-BUdR DNA before shearing was due to the dilution of BUdR by thymine pool in spores or that ^{3}H-BUdR was incorporated by repair synthesis. These results indicated that ^{3}H-BUdR DNA was indeed a hybrid DNA of a small molecular weight of about 2×10^6, and that it is joined covalently to a light DNA of about 20×10^6 M.W. It might be possible that DNA replication was initiated and a significant amount of DNA had been synthesized before the addition of ^{3}H-BUdR. Therefore DNA synthesis during thymine starvation was determined by incorporation of ^{32}P

into DNA. A significant amount of ^{32}P was incorporated into DNA during the thymine starvation. The amounts of ^{32}P-labeled DNA, however, varied depending on both the length of the starvation period and age of spores that were kept in H_2O at $4^{\circ}C$. They ranged from 1% to 0.05% of the DNA in the spores. The density of ^{3}H-BUdR DNA was independent of the amount of ^{32}P incorporated during thymine starvation. It was also found that alkali treatment separated ^{3}H-activity from ^{32}P activity in CsCl density gradients.

From these results we have constructed a replication model in which newly synthesized strands of two daughter chromosomes are covalently joined to the terminus of the parental chromosome.

Induction of Phage-like Particles and Bacteriocins in Bacillus Subtilis Strain 168

In bacteria the development of prophages and bacteriocins may be induced by a variety of agents. The bacteria may or may not be lysed during induction. Ultraviolet radiation, hydrogen peroxide, 5-bromodeoxyuridine, mitomycin C and thymineless growth have been used for induction.

Spores of a thymine-requiring mutant of B. subtilis were germinated in a thymineless synthetic medium. Following lysis with lysozyme and preparative centrifugation in a CsCl density gradient a fraction was isolated in which many phage-like particles were detected under the electron microscope. In a thymineless medium germinated spores show no evidence of lysis or thymineless death for 20 hours, as determined by optical density and colony count.

Treatment of the same cells with 4 — 5 μg/ml mitomycin C during vigorous exponential growth caused lysis of the entire culture within 2 hours. Electronmicroscopic observation of the lysate reveals the following features (see Figures 1a and 1b):

1. Phage-like particles with extended tail sheath (p) and with contracted tails (c).

2. Numerous tails, some extended (te), some contracted (tc).

3. Thread-like objects, many microns long and about 6A thick.

4. At least four kinds of phage heads, some full, some empty, as determined by their appearance after 1.8% phosphotungstic acid (pH 6.7) staining.

5. One also observes tail-sheath sections in cross-section (ts). Six subunits lie peripherally to form the sheath.

There is a similarity between the complete (extended) phage tail, the free tails and the long threads. Contracted tails are thicker than the long threads, whereas some tails seem to be losing their outer sheath, giving rise to the peculiar subunit structure of the tail core. These subunits are also seen along the long threads. Some of the long threads hold empty or full heads either at the end or somewhere along the threads (see arrows). The heads are always connected to the thread at a point between two subunits. In one case a thin neck links a long thread with a head (see left center of figure 1a), as is the case in complete phage-like particles.

Differential centrifugation and density gradient centrifugation in CsCl of the crude lysate yielded four different fractions. The long threads were separated from the smaller particles by spinning the crude, concentrated lysate for one hour at 20,000 rpm in a Spinco No. 30 rotor. The supernatant yielded three discrete bands in CsCl (1.35 gm/cc). Band 1 with a density of 1.37 gm/cc contained complete phage-like particles (see figure 2a). Band 2 of density about 1.33 gm/cc was composed of tails and empty headed phage-like particles. Band 3 contained material which has not yet been identified.

A technique has been devised to assay the killing activity of these particles. All three particles; phage-like particles, tails and the long threads, showed killing activity on B. subtilis strain 168.

Because of the similarity in both the structure seen by electron microscopy, and biological activities between the tails, the long thread and known bacteriocins, we propose to call the tails and the long threads subticin and polysubticin respectively.

Protein Synthesis During the Early Stage of Germination of B. Subtilis

Previous experiments with B. subtilis spores showed that inhibition of protein synthesis during a critical period in the replication cycle of DNA reduced the incorporation of radioactive thymidine into DNA. The experiments indicated that some protein, whose synthesis is inhibited by chloramphenicol, is synthesized at each cycle and initiates DNA replication.

Further experiments have supported this conclusion. The rates of synthesis of protein, RNA, and DNA during germination were estimated by exposing the spores to pulses of radioactive leucine, thymidine, and uracil and measuring the incorporation of radioactivity into the TCA insoluble fraction. The rate of leucine-1-C^{14} incorporation gradually increased to a small peak at 2 hr 20 min, declined briefly, and thereafter increased rapidly to high values. The rate of ^{3}H-thymidine incorporation indicated that DNA replication was completed by 2 hr 50 min in the first generation and by 3 hr 50 min in the second generation. These results suggested that a protein or small number of proteins was formed just prior to the initiation of DNA replication in the first generation. As expected the rate of RNA synthesis underwent more complex changes. The results, however, indicated that some RNA was formed just prior to the formation of the early protein. The formation of early RNA and early protein was not affected by the absence of thymidine. These results indicated that early RNA and the early protein were formed independently of DNA replication. The rates of synthesis of proteins and RNA after 3 hrs were greatly reduced in the absence of thymidine.

The present aim of this project is to isolate the early protein. Preliminary experiments indicated that sonication and homogenization with glass beads do not break up the partially-germinated spore. Some labelled protein, however, was extracted from protoplasts of labelled spores.

Future Plans

Further Studies on Initiation of DNA Replication

Mechanism of initiation of DNA replication will be further investigated in two directions.

The first is based on the finding that newly-replicated strands may be joined covalently to the ends of the parent chromosome. If this model is correct, one should expect the existence of a unique structure at the initiation point. Attempts will be made to isolate such a structure both from spores and germinated spores. A method was devised to prepare spores in which the chromosome was labelled over a length representing less than 1% of the distance from the initiation point. Physico-chemical, chemical and biological characteristics of the DNA isolated from these labelled spores will be determined.

The second line is the isolation and characterization of the proteins synthesized during an early stage of germination prior to the initiation of DNA replication. In addition to kinetic studies of the pattern of protein synthesis and the initiation of DNA replication, we will try to assay the initiation of chromosome replication directly using *in vitro* DNA synthesis system of partially broken protoplasts. Comparisons of these early proteins with the serine-rich protein isolated as DNA-protein complex will also be made. We have several mutants of *B. subtilis* whose spores are incapable of germinating at higher temperatures. These mutants may be also useful for studying the initiation of DNA synthesis during germination.

Further Studies on Bacteriocins in B. subtilis

Particles induced by mitomycin C or thymineless germination in *B. subtilis* are closely related both in the structure seen by the electron microscope and in their biological activity. They are on one hand very similar to well known bacteriocins, colicin and pyocin, and on the other hand very close to lysogenic phages. We shall determine the physico-chemical and chemical structures of these particles along with the nature of their biological activity.

We expect that these investigations will lead to a clear understanding of the relation between induction of prophages and biosynthesis of bacteriocins by episomes.

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FIGURE 1a, 1b. Crude lysate (concentrated in 1% ammonium) acetate of mitomycin-C treated B. subtilis 168 LTT. Magnification: (1a) 60,000x; (1b) 100,000x.

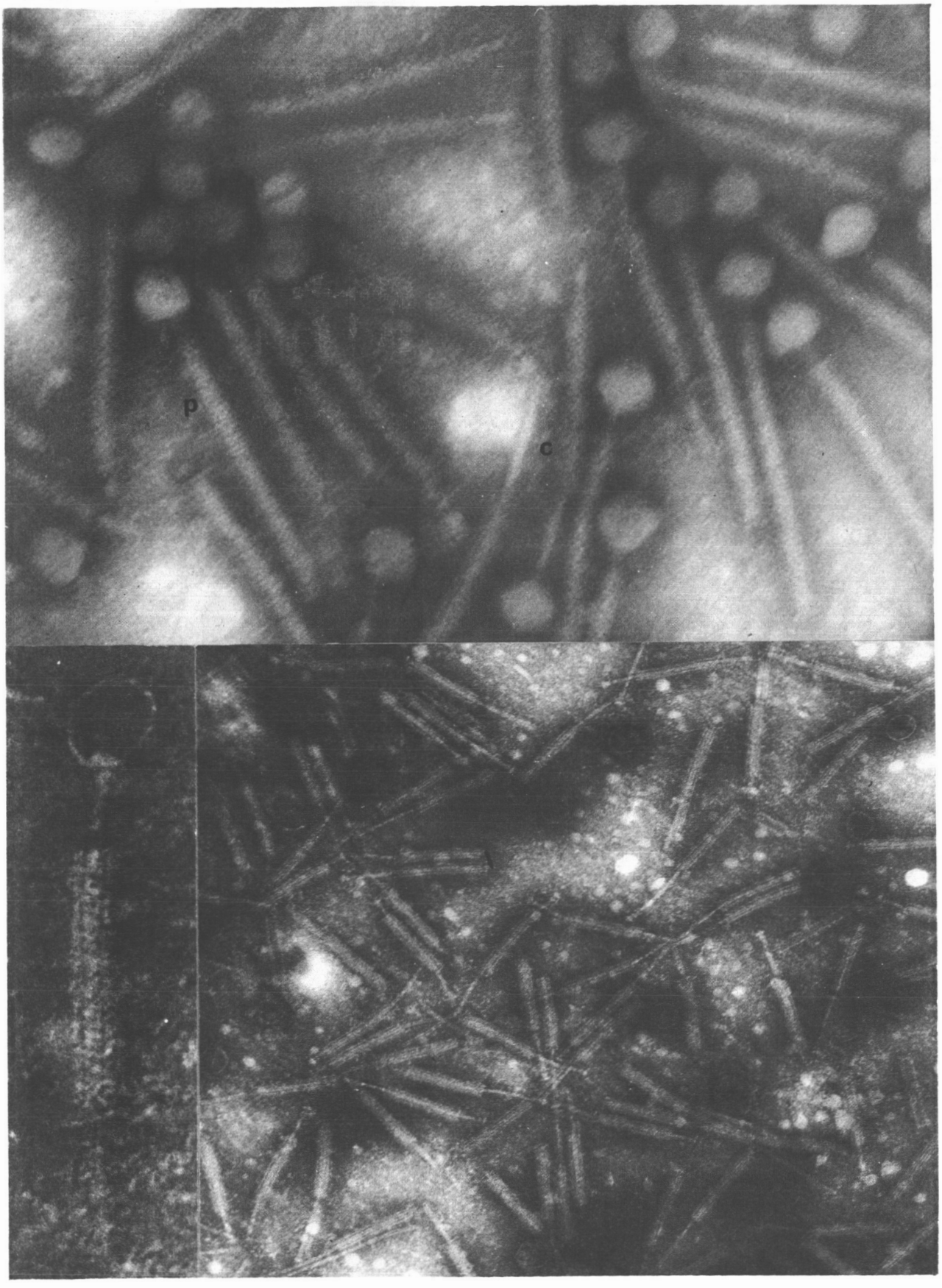

FIGURE 2a, 2b. Purified preparations (bands 1 and 2, see text) of phage-like particles and subticin. Magnification: 2a: 300,000x; 2b: x125,000. Inset: x400,000.

SEQUENTIAL STUDY OF SPINACH FERREDOXIN

(H. Matsubara, R. Sasaki, D. Ouye and J. Nakao; in collaboration with the Department of Cell Physiology)

The complete amino acid sequence of spinach ferredoxin has been elucidated. Tryptic digestion of S-β-aminoethylcysteinyl-ferredoxin, chymotryptic digestion of S-carboxymethylcysteinyl-ferredoxin (CMFd) and thermolysin digestion of CMFd and oxidized ferredoxin yielded a complete overlapping sequence of spinach ferredoxin as shown in Figure 1. There was a discrepancy between the composition obtained from direct analyses and that deduced from the sequence. However, this was overcome by developing a new purification procedure for spinach ferredoxin on a hydroxylapatite column which gave a composition by total analysis identical with that derived from the sequence. Methionine was absent. Molecular weight determinations by gel-filtration and terminal analyses supported the results of the sequence which gave a molecular weight of approximately 10,650 including 2 irons and 2 labile sulfurs. We showed that thermolysin was very effective for obtaining peptide fragments of various sizes and degradation with this enzyme adds another powerful tool to protein chemistry. Spinach ferredoxin has 5 cysteine residues and 3 of the 5 cysteines are near the center of the molecule. Two others at residues 18 and 77 are approximately equidistant from 47. A comparison of the amino acid sequences of spinach and clostridial ferredoxins showed regions of similarity which revealed homology over a distance of 19 consecutive amino acid residues, 42-60 in spinach and 9-27 in Clostridium butyricum ferredoxin. This homology suggests an evolution in both types of ferredoxins from a common archetype. Other indications of homology were also suggested. As previously pointed out, it was found that a repetitive sequence, 1-9 and 78-86, suggested an event of lengthening by genetic recombination in an early stage of evolutionary history. Many acidic amino acid clusters were found and several hydrophobic regions are present. The locations of the cysteine residues and the fact that spinach ferredoxin has only 2 iron atoms and 2 labile sulfurs both indicate that spinach ferredoxin has an electron transfer mechanism and a molecular structure around cysteine residues differing from those in the clostridial ferredoxins.

Future Work Planned

1. Other ferredoxins from photosynthetic organisms will be purified and subjected to sequential study to explore the relation of structure to function of photosynthetic ferredoxin and to continue our evolutionary studies of ferredoxin.

2. Structural study of ribosomal protein fractions will be carried out with the cooperation of Dr. K. Hosokawa.

3. Sequential studies of thermolysin will be continued.

1 10 20

NH_2-Ala-Ala-Tyr-Lys-Val-Thr-Leu-Val-Thr-Pro-Thr-Gly-Asn-Val-Glu-Phe-Gin-Cys-Pro-Asp-

30 40

Asp-Val-Tyr-Ile-Leu-Asp-Ala-Ala-Glu-Glu-Glu-Gly-Ile-Asp-Leu-Pro-Tyr-Ser-Cys-Arg-

50 60

Ala-Gly-Ser-Cys-Ser-Ser-Cys-Ala-Gly-Lys-Leu-Lys-Thr-Gly-Ser-Leu-Asn-Gln-Asp-Asp-

70 80

Gin-Ser-Phe-Leu-Asp-Asp-Asp-Gin-Ile-Asp-Glu-Gly-Trp-Val-Leu-Thr-Cys-Ala-Ala-Tyr-

90 97

Pro-Val-Ser-Asp-Val-Thr-Ile-Glu-Thr-His-Lys-Glu-Glu-Glu-Leu-Thr-Ala-COOH

FIGURE 1

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OPTICAL ROTATORY DISPERSION, X-RAY SCATTERING AND BIREFRINGENCE OF DNA AND RNA

(Marcos F. Maestre and Kimmy Sieux)

Optical Properties and Denaturation Properties of Nucleic Acids in High Salt Solutions

The nucleic acids investigated were the following: T2 DNA, T7 DNA and R17 RNA. The DNAs were prepared by the phenol extraction method and the R17 RNA was a gift from Professor Kaesberg of the University of Wisconsin.

The salts used in the study were LiCl in concentrations ranging from 0.4% to 24.6% buffered to pH 7.2 with Tris-HCL, and sodium acetate, saturated (around 12 molar) buffered to pH 7.0.

The physical states of the nucleic acids were followed from changes in the optical properties as a function of the salt concentration and temperature. The changes in the ORD spectra were of a radical nature as shown by the disappearance of the 290 mμ peak of the DNA in both T2 and T7 DNAs and reduction of the 260 mμ as the salt was increased to concentrations of 6 molar for LiCl and 12 molar for sodium acetate. The alteration of ORD spectra in the temperature studies show that as expected there is a significant change when the DNA is denatured. By contrast the optical properties

of the single-stranded R17 RNA remains unchanged in the high salt concentration and its thermal behavior differs little from that of the same molecule at low salt concentrations. A reasonable interpretation of the data is that the double stranded structure of the nucleic acids is unusually sensitive to variations in salt concentration above 1-molar. The results may mirror a change in: 1) the geometries of the bases and the base-to-base interactions, or 2) the optical properties of the bases as changed by a radical alteration in polarity of the solvent, or 3) by both effects in varying amounts.

The next objective of the research program is the study in detail of the influence of base composition of the ORD spectra and the effect of variation of salt cations and anions on the thermal behavior of the DNA and RNA. This research should be concluded in the next six months of the program.

The Measurement of the Helical Content of Fully Oxidized Spinach Ferredoxin

(Marcos F. Maestre and Hiroshi Matsubara)

Spinach ferredoxin has an abundance of Cotton effects in the visible part of the spectrum.[2] This precludes the use of the Moffit-Yang equation for the determination of its helical content. However, the helical content of proteins has been correlated with the specific rotations at both 233 mμ and 198 mμ.[3] It is expected that the measurements in that range of rotations will be reasonably unaffected by the rotations in the visible range of the spectrum ($\lambda \geq 400$ mμ) and the Cotton effects at about 300 to 400 mμ. With the reservation that the specific rotations can be influenced by the higher wave-length rotations, the following values were obtained from the Cotton effect of the peptide chromophore at 212 mμ. $\alpha_{228 m\mu}$ gave a value of 19% helicity and $\alpha_{198\ m\mu}$ gave a value of 26% α-helical content of the ferredoxin molecule. These approximate values of the helical content seem to indicate that the molecule is of low α-helicity. Further work on the reduced form of the ferredoxin molecule is expected to yield more information on the subject.

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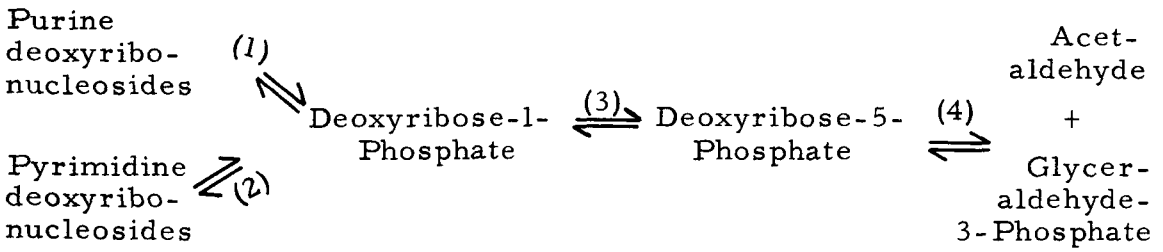
REGULATION OF DEOXYRIBOSE METABOLISM IN E. COLI

(H. O. Kammen, E. E. Turner, M. Strand and S. Spengler)

The degradation of deoxyribosyl derivatives in E. coli involves a group of enzymes which function in the formation, interconversion and cleavage of deoxyribose phosphates, as shown below:



The enzymes which participate in this pathway include (1) purine deoxyribonucleoside phosphorylases, (2) thymidine phosphorylase (TP), (3) phosphodeoxyribomutase (PDM), and (4) deoxyribose-5-phosphate aldolase (DPA).

We have found that at least three of these enzymes, TP, PDM, and DPA, are jointly induced when E. coli B and other strains are grown in the presence of deoxyribonucleosides. This induction occurs with any of the commonly-occurring deoxyribonucleosides, but not with free deoxyribose, suggesting that deoxyribose-1-phosphate and/or its metabolites serve as the intracellular inducers. This belief is supported by the finding that mutants of E. coli B which are defective for TP are induced for PDM and DPA by purine deoxyribonucleosides, but not by thymidine. Similarly, growth of E. coli B (a thymine auxotroph) or its prototrophic revertants in thymineless media leads to the induction of TP and PDM without the necessity for external inducers. These strains lack DPA, as noted in earlier reports.

These induction patterns provide a convenient starting point for the characterization of PDM, which has not been previously described. Two sensitive assay methods have been devised for this purpose. An isotopic assay, which employs deoxyribose-1-^{32}-P as substrate, measures its conversion to acid-stable ^{32}P. A spectrophotometric assay is also useful with extracts from bacterial strains which lack DPA. Incubation of deoxyribose-1-P with such extracts leads to the accumulation of deoxyribose-5-phosphate as the primary product of the mutase activity; the amounts of deoxyribose-5-P produced are quantitated by coupling the reaction products with DPA, under conditions which lead to the oxidation of DPNH.

PDM activity in dialyzed crude extracts is optimal at pH 8.5–9.0 and is stimulated maximally (3X) by catalytic concentrations of glucose-1, 6-diphosphate. No additional Mg^{++} is required, and phosphate ion is inhibitory. At least 85% of the reaction products can be accounted for as deoxyribose-5-phosphate, on the basis of chemical, chromatographic and enzymatic properties. Our initial attempts to demonstrate reversibility of the reaction have not been successful.

On the basis of induction patterns, the PDM activity appears to be distinguishable from phosphoglucomutase. Other related enzymes have been examined during the induction of E. coli B. Preliminary results suggest that low-level induction of deoxyguanosine phosphorylase may occur under these conditions; the levels of deoxycytidine deaminase are unchanged. Deoxyadenosine deaminase is induced in the presence of purine deoxyribonucleosides, but not of thymidine; thus, this enzyme is probably under a different regulatory control than TP, PDM, and DPA.

Our plans for the immediate future include (a) Purification and further characterization of the reaction mechanism of PDM; (b) Further inquiries into the possibility that other enzymes are jointly induced; (c) Isolation and characterization of E. coli mutants which are defective for the enzymatic functions described, as a preliminary to determining whether the formation of these enzymes reflects the functioning of a single genetic system.

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AZOTOBACTER VINELANDII RNA POLYMERASE. STUDIES ON THE RIBONUCLEOSIDE TRIPHOSPHATE BINDING SITE

(J. Krakow, W. Horsley, M. Karstadt, R. Siegel, E. Fronk)

RNA synthesis involves ribonucleoside triphosphate selection and binding by RNA polymerase followed by phosphodiester bond formation with pyrophosphate release. The exception to this mechanism occurs during the initiation of a new RNA molecule where the first nucleotide in the RNA chain is incorporated as the unaltered ribonucleoside triphosphate. The general nature of chain initiation by the A. vinelandii RNA polymerase is very similar to that found with the E. coli RNA polymerase by Maitra and Hurwitz (1965). We have recently found that the A. vinelandii RNA polymerase binds ribonucleoside triphosphates under conditions where RNA synthesis is prevented. The assay procedure involves mixing the enzyme with a ribonucleoside triphosphate labeled with ^{32}P in the γ position (e.g. AMPP^{32}P) and passing the complex through a column of Sephadex G 75 which enables one to separate the polymerase nucleotide complex from unbound nucleotides. Native RNA polymerase will bind ribonucleoside triphosphates while heat-denatured enzyme is unable to bind. Preliminary studies have shown that at 5° ATP, GTP, CTP or UTP will bind to about the same extent, at 30° the affinity of the enzyme for the pyrimidine nucleotides, CTP and UTP decreases whereas there is little change with regard to complex formation with ATP or GTP. The GMPP ^{32}P bound to polymerase can be recovered as GMPP ^{32}P after ion exchange chromatography, showing that no alteration of the nucleotide has occurred as a result of binding to the enzyme and that diester bond formation has not occurred.

Further studies on the mechanism of ribonucleoside triphosphate binding will be concerned with whether the presence of various template polymers confers specificity on the nucleotide binding reaction. This is obviously of great interest and experiments are planned to test this. A more thorough study of the effect of temperature on the stability of the nucleotide triphosphate polymerase complex is planned as well as the effect of ionic strength on the binding reaction. The effect of temperature on incorporation of ribonucleoside triphosphates into the 5'-termini of RNA will be studied to see

whether the binding reaction can be correlated with chain initiation and whether the ratio of pyrimidine to purine termini will increase as the incubation temperature is decreased. It is hoped that these studies can be used to determine the number of nucleotide binding sites on polymerase and especially an elucidation of the role of the template in the mechanism of nucleotide selection.

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THE DETERMINATION OF BASE SEQUENCES IN TMV-RNA

(S. Mandeles, F. J. Fearney, M. L. Larsen, M. Kamio)

The work is proceeding along three lines: base sequence experiments, mapping experiments, and formation of sub-units. To date, the base sequence experiments have yielded data that support GpCpCpCpA as the sequence for the 5'-linked end of TMV-RNA. These data were obtained through use of semicarbazide- ^{14}C as a label for that end of the molecule. However, owing to the instability of this label under the conditions of isolation and purification, a different, more stable label is being sought. Among the various possibilities are: reduction of the bis-semicarbazone with $NaBH_4$; use of ^{14}C labeled isonicotinic acid amide; or formation of stable boronic acid complexes. Development of a more stable label will permit continuing application of the sequence determination procedure.

In the mapping experiments as described in the previous report, a number of unique oligomers have been isolated from a T_1 -RNase digest of TMV-RNA. The longest of these appears to have

a chain length of 60 bases. These data were obtained by isolation of the long oligomer (after chain length separation chromatography) and digestion with pancreatic RNase. These digests were then separated by two-dimensional chromatography-electrophoresis on paper and the individual small oligomers were identified and measured spectrophotometrically. Work is continuing on the determination of the base sequences in this oligomer. It is planned to locate the position of this oligomer in the intact TMV-RNA molecule. Studies are being made on the binding of oligomers of known base sequence of TMV-RNA in considering the question of formation of sub-units in TMV-RNA. The possibility that TMV-RNA is composed of hydrogen-bonded sub-units was investigated by comparing the sedimentation behavior of TMV-RNA in dilute Tris-buffer with the same TMV-RNA in buffered 7M urea. Both velocity and equilibrium sedimentations were performed. The data indicated that there were no hydrogen bonded sub-units that are susceptible to cleavage in 7M urea.

Publications

1. Mandeles, S. and E. C. Woods, Continuous Countercurrent Dialysis of Large Volumes. Anal. Biochem., 15, 523 (1966).

2. Mandeles, S. and C. R. Cantor, Base Composition of Intact Nucleic Acid Oligomers. Biopolymers, 4, 759 (1966).

3. Mandeles, S. and G. Bruening, Preparation of TMV-RNA. Biochemical Preps., Vol. 12 (in press).

4. Mandeles, S. and H. Kammen, Use of Charcoal for Isolation and Purification of Nucleic Acid Oligomers. Anal. Biochem., 17, 540 (1966).

DEVELOPMENTAL BIOCHEMISTRY

(J. Hosoda, E. Mathews)

Future Plans

1. Assembly of head of T4 phage in relation to DNA synthesis.

It is assumed that the presence of DNA as a core is necessary for coat proteins to assemble and to make the head. We found mutants in gene 30 that do not accumulate DNA but

make fairly large amounts of head proteins. We also found a sensitive method of detection of head protein integration into solid structures. We wish to use these methods to find out if DNA is necessary for coat assembly.

2. Analysis of DNA structure made by mutants in gene 30.

Amber mutants in gene 30 of T4 make a small amount of DNA which is later degraded by enzyme(s) induced by T4 itself. Induction of synthesis of late proteins of T4 is dependent upon T4 DNA synthesis but the mechanism is not known. Amber mutants in gene 30 allow induction of late proteins while an amber mutant in gene 56, which also has an unstable DNA, does not. Analysis of these DNAs and comparisons of them with each other may lead to understanding the mechanism by which late protein synthesis is induced.

3. Analysis of phage-induced proteins associated with the bacterial cell wall and gel electrophoresis containing SDS.

Alterations of cell wall character have been observed after infection with T-even phages. We found that a considerable portion of newly-synthesized protein after infection was bound to the cell wall-membrane fraction and could not be analyzed in regular acrylamide gels. It was found in Levinthal's laboratory that treatment of the cell wall with SDS, followed by use of acrylamide gel containing SDS was effective in analysis of E. coli cell wall protein. Phage-induced proteins associated with cell wall-membrane fraction will be analyzed by this technique.

4. A general survey of the pattern of protein synthesis after infection of T4 mutants concerned with early function will be made.

ACTION OF VARIOUS MUTAGENS ON TMV AND TMV-RNA

(B. Singer, H. Fraenkel-Conrat; In collaboration with the Department of Molecular Biology)

When N-methyl-N'-nitro'N'nitrosoguanidine (MNNG) is allowed to act on tobacco mosaic virus (TMV) RNA at about 25° and pH 4.5-9, the viral infectivity rapidly decreases. The inactivation kinetics are complex due to decomposition of the reagent, which is pH dependent. The reaction is mutagenic, in terms of local lesion response on N. sylvestris, though at a much lower level than that of nitrous acid or hydroxylamine. When the reagent acts on intact TMV at pH 5.5, the rate of inactivation is much slower but the mutagenicity is high, even compared to that of nitrous acid. Thus a 37-fold increase in mutagenicity was obtained with RNA isolated from treated virus which retained 44% of its original infectivity. Mutagenicity decreased at pH 6.5 and much more at pH 7.5. When extensively MNNG treated RNA is degraded with snake venom diesterase and chromatographed two-dimensionally, less pG is found than in control RNA, and a fifth spot of striking whitish fluorescence is noted. If the treated RNA is hydrolyzed with N HCl, a similar loss of G and a lesser decrease in Cp are observed. It appears possible that in the intact virus, as contrasted to the RNA, the reaction with C is greatly favored over that with G, and that the former represents a highly mutagenic and the latter a lethal event.

Future Plans

We plan to continue the investigation of the mechanism of action of chemical mutagens in producing changes in tobacco mosaic virus RNA.

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