Space Sciences Laboratory
University of California
Berkeley, California 94720

Semi-Annual Report on

CHEMISTRY OF LIVING SYSTEMS

Supported by

NASA Grant
NsG 479

For the period

April 1 through September 30, 1967

Principal Investigator — Professor Thomas H. Jukes

Space Sciences Laboratory Series 8, Issue 91

September 30, 1967

CHEMISTRY OF LIVING SYSTEMS

NASA Grant
NsG 479

STUDIES OF DNA REPLICATION IN *Bacillus subtilis*

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

DNA SYNTHESIS BY GERMINATING SPORES OF *Bacillus subtilis*

I. MECHANISM OF INITIATION OF DNA REPLICATION

In the preceding report we described our finding that newly synthesized DNA strands were covalently joined to pre-existing DNA in the spores of *B. subtilis*. Based on this, a circular replicating chromosome was proposed as a model for initiation of chromosomal replication. Subsequent experiments have confirmed this. A thymine-requiring spore was prepared in a medium containing D_2O and ^{15}N, and was germinated in H_2O and ^{14}N medium with ^{3}H-thymidine. In this procedure pre-existing DNA strands are labeled with D and ^{15}N, thus having heavier density, and newly synthesized strands should have a normal light density. DNA was isolated and densities in CsCl were measured. The results showed that a DNA strand containing ^{3}H was joined to a heavy strand containing D and ^{15}N. This result clearly indicated that newly-synthesized DNA strands were covalently joined to the pre-existing DNA, thus confirming the previous finding. We are going to study physico-chemical properties of the initiation region to establish the proposed structure directly.

II. DNA REPLICATION WITH 5-BROMODEOXYURIDINE

Use of 5-bromodeoxyuridine (5-BUdR) is important in studying DNA replication. However, chromosomal replication with 5-BUdR has not been studied carefully. We have, therefore, studied the

mode of chromosomal replication with 5-BUdR during germination of thymine-requiring spores of *B. subtilis*.

P^{32} labeled spores of *B. subtilis* (leu$^-$, try$^-$, thy$^-$) were germinated in the absence of thymine and replication of the chromosome was initiated by adding H^3-5-BUdR. DNA was isolated at various times and centrifuged in CsCl. The amounts of heavy (both strands labeled with 5-BUdR), hybrid (one of the two strands labeled with 5-BUdR), and light (both strands normal) DNA were measured from the profiles after fractionating into 70 fractions. The results are in *Figure 1*. Re-initiation of chromosomal replication took place when when the primary replication had proceeded for only 3% of the total chromosome. It was also shown that two chromosomal forks were re-initiated at about the same time. Furthermore, the rate of the secondary replication is about the same as that of primary replication. During germination with 5-BUdR, protein synthesis was severely inhibited and the cells eventually died. The transforming activity of heavy DNA was less than 1% of that of hybrid or light DNA. It might be possible that a complete replacement of thymine by 5-BU would not only make DNA less functional due to incomplete complementation between 5-BU and adenine but also would cause physical instability of the chromosome. Such instability might be the primary cause of death and of non-function in transformation. We are therefore going to study physical properties of 5-BU DNA in comparison with its transforming activity. The mechanism of re-initiation will be also studied with this system.

AN INDUCED PHAGE-LIKE PARTICLE OF *Bacillus subtilis*

In the last semi-annual report, we reported the existence of phage-like particles in the lysate of *B. subtilis* treated with mitomycin C during the logarithmic growth phase. The method of isolation and purification of various particles was reported. In this report, we describe physical and biological properties of the phage-like particle and of its PBSH DNA (PBSH = Phage *B. subtilis* H).

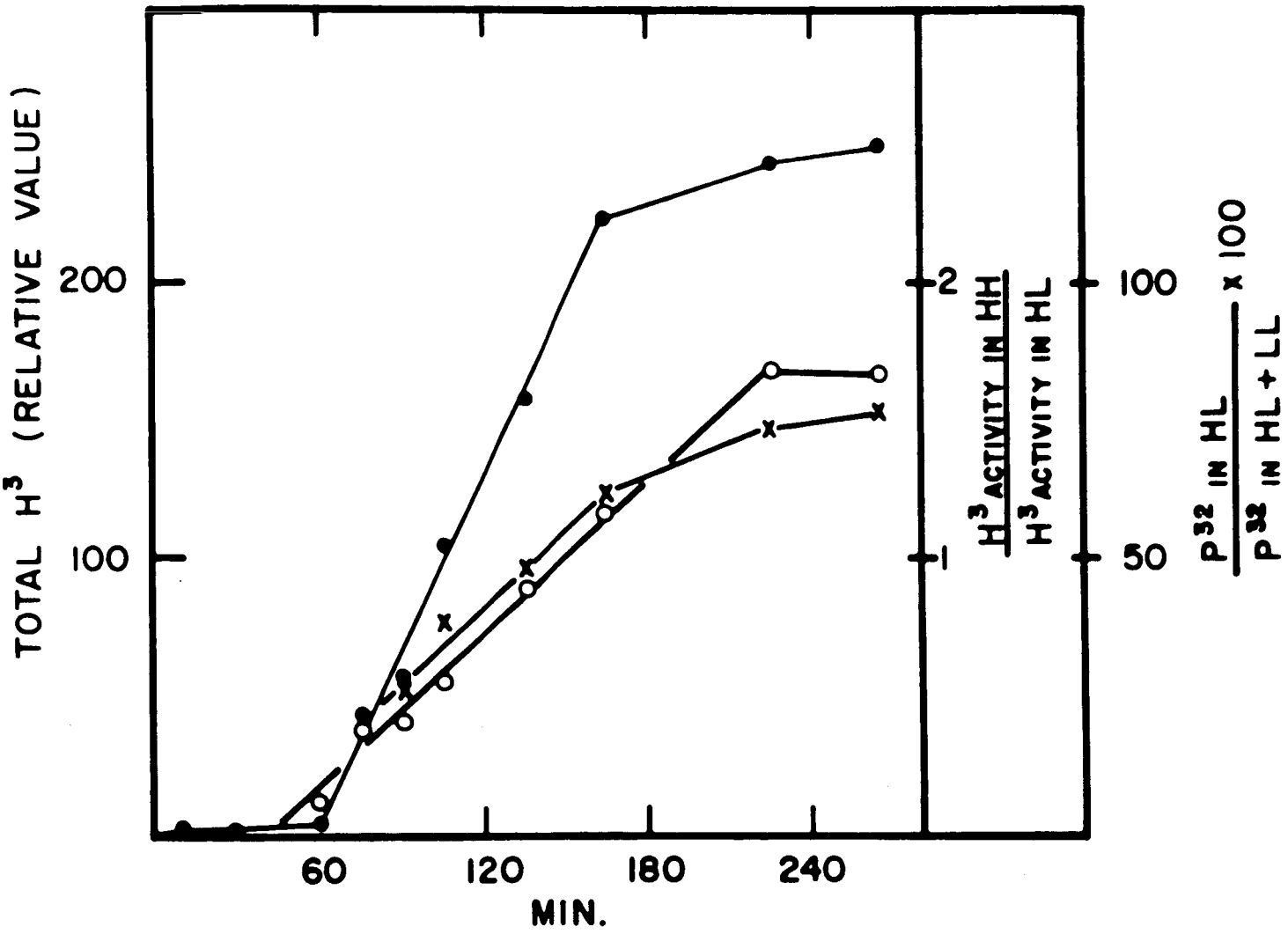

Figure 1. Chromosomal replication with 5-BUdR. P^{32}-labeled spores were germinated in the absence of thymine for 4 hrs and H^3-5-BUdR was added at time 0. Heavy (HH), hybrid (HL), and light (LL) DNAs were fractionated by centrifugation in CsCl.

●—● Total amount of H^3-activity in HH and HL, relative to the value at 105 min.

○—○ $\frac{H^3 \text{ in HH}}{H^3 \text{ in HL}}$ represents degree of re-initiation

×—× $\frac{P^{32} \text{ in HL}}{P^{32} \text{ in HL + LL}}$ represents % of primary replication

I. PHYSICAL PROPERTIES

A. Sedimentation Constants *(S)*

The S of the particle was determined by band sedimentation in CsCl of various densities, ranging from 1.05 to 1.35 gm/cc. Extrapolation of S to unit density gave a sedimentation constant of $S_0 = 248$.

DNA was extracted from the particle by gently shaking with phenol at room temperature. The S of the DNA was measured following various treatments. The results were as follows:

Sedimentation constants and density of PBSH DNA

	Native DNA	Heat denatured DNA*	Renatured DNA**
$S_{20,W}$	22.1	34.8	heterogeneous
Molecular weight	8.7×10^6	4.7×10^6	heterogeneous
Density in CsCl	1.709	1.724	1.713

*Heat denatured at 100° for 10 min. and quickly cooled in ice.

**Incubated at 65° for 24 hrs. in SSC (Std. Sodium Citrate).

B. Base Composition of DNA

No difference in density was detected between DNA from PBSH and *B. subtilis* DNA in CsCl or $CsSO_4$ density gradients. The melting temperatures of the two DNAs were also measured to detect any differences in base composition, but no differences could be detected between the two within the limits of sensitivity of the method. The base composition of PBSH DNA was calculated as 43 ± 2.4% GC by the above methods.

C. Electron Microscopy

The contour length of PBSH DNA was determined by means of the Granboulan and Nirelean modification (*J. Microscopie*, *6*:23, 1967) of Kleinschmidt's technique. The results confirmed the preceding findings for molecular weight. (*Figure 2*).

The number of PBSH liberated per induced cell was determined by electron microscopy. Calibration values were established between the concentration of PBSH in solution and its optical density at 260 mμ. Thus $O.D._{260} = 1$ corresponded to 6.25×10^{11} particles/ml.

II. BIOLOGICAL PROPERTIES

A. Double-Label Experiments

B. subtilis cells were labeled for 4–5 generations with H^3-thymidine prior to induction and labeled with P^{32} during induction by mitomycin C. Analyses of the isotopes in PBSH DNA revealed that 14.6% of PBSH DNA originated from the cellular DNA labeled prior to induction and 40% of DNA present in the cells at the time of induction found its way into PBSH DNA. The total DNA content of the induced culture increased 2.7-fold during the induction period. Studies are in progress to determine whether this increment of DNA was due to DNA synthesis by cells or multiplication of PBSH.

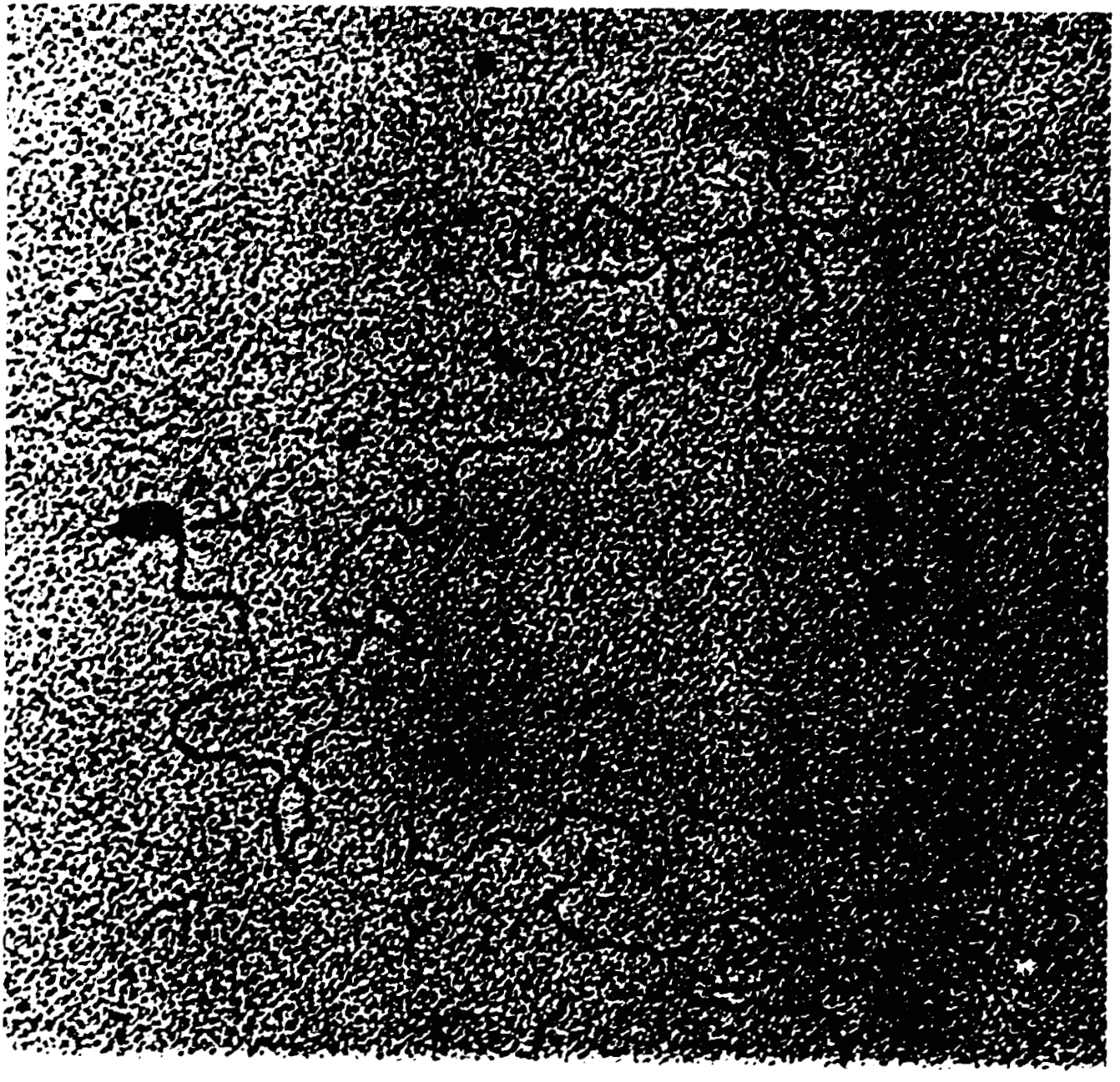

Figure 2. Electron photomicrograph of PBSH DNA. Uranium shadowing. Magnification 150,000. Total length 36 μ which corresponds to 7.2×10^6 daltons.

B. Transduction by PBSH and Transformation by PBSH DNA

PBSH transduced various mutant derivatives of *B. subtilis* strain 168. Under the conditions used, the efficiency of transduction is between 1.5×10^{-6} and 6.2×10^{-8} adenine transductants per PBSH particle (about 8,000 transductants/μg DNA). The transducing efficiency of ade-16 marker, which is located closest to the origin of the *B. subtilis* chromosome, was between 25 to 70 times higher than that of any other marker. This suggested that PBSH is closely linked specifically to the ade-16 marker or to the origin of the *B. subtilis* chromosome.

DNA extracted from PBSH transformed 168 mutants with somewhat lower efficiency than was obtained by transduction; about 4,000 ade-16 transformants/μg DNA. Again, transformation efficiency of ade-16 was higher than that of any other marker tested.

The gene frequency of the culture was measured during induction in order to study the mechanism of PBSH induction. The results indicated that there was a 2- to 3-fold increase in specific activity for ade-16 marker while those for other markers decreased 2- to 3-fold.

As a consequence, the ratio of ade-16 to other markers gradually increased to approximately 6 during a 60 min. induction period. Experiments of this type are in progress to determine whether or not PBSH DNA replicates autonomously during induction.

A preliminary result indicated that the PBSH particle which transduced ade-16 marker had a heavier density in CsCl, indicating that the particle has approximately 10% more DNA than other particles.

STUDIES ON RNA POLYMERASE OF *Azotobacter vinelandii*

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

The synthesis of RNA *in vivo* is catalyzed by the enzyme RNA polymerase using DNA as the template. Because of its crucial role in the existence and maintenance of life, RNA polymerase has been a

subject of great interest. We have been studying the enzyme in cell-free systems. Recently our attention has turned to the synthesis of polyribonucleotides in the absence of template. Such reactions are of interest in the problem of chemical evolution since we may assume that polymerizing "protoenzymes" existed at a time when neither template DNA nor polyribonucleotides existed. It is of interest to see what the requirements are for the *de novo* synthesis of polymers and also whether any order or sequence is imposed in the product even though no template has been added. It has been shown that unprimed synthesis of poly A:poly U will take place with the *E. coli* RNA polymerase. Using the RNA polymerase purified from *Azotobacter vinelandii*, we have confirmed this finding and have extended these studies by demonstrating that an alternating copolymer of IMP and CMP is synthesized from ITP and CTP in the absence of primer.

All unprimed reactions catalyzed by DNA and RNA polymerase are characterized by a lag period of variable length. Under conditions of saturating ATP and UTP, the lag period for rA:rU synthesis is 15 min. at 37°. At 27°, the lag is over 60 min., while at 17° very little polymer is synthesized even at 150 min. incubation. During the lag period, one may assume that the synthesis of oligonucleotides with a sequence identical to the polymer formed subsequently in the reaction takes place and that this initiation process is very temperature-sensitive. When a reaction mixture is incubated at 37° for 10 min. and then the temperature is lowered to 17° extensive rA:rU synthesis occurs showing that the formation of oligomers has taken place in the first 10 min. at 37° and that subsequent chain elongation and transcription will occur at 17°. It is also possible to shorten the lag phase at 17° by adding an oligomer of 5 adenylic residues (ApApApApA).

Although it has been possible to synthesize rA:rU in unprimed RNA polymerase reactions, attempts to obtain polymerization of CTP and GTP in unprimed reactions were unsuccessful. We found that ITP, the Watson-Crick analogue of GTP, was incorporated into a polymer in

the presence of CTP. The polymer synthesized in the unprimed reaction is the rIC copolymer in which the IMP and CMP residues are alternating. The alternating structure was determined by nearest-neighbor analysis using $\alpha^{32}P$ -CTP and $\alpha^{32}P$ -ITP. When poly C is added only poly I and poly C are formed.

REACTION	$\alpha^{32}P$-ITP + CTP		$\alpha^{32}P$-CTP + ITP	
	SEQUENCE	CPM	SEQUENCE	CPM
A Unprimed	CpI	10140	CpC	107°
	IpI	48	IpC	17041
B poly C-primed	CpI	37	CpC	13497
	IpI	10757	IpC	87

The inability of GTP to replace ITP in the reaction is unexpected since GTP is readily polymerized to poly G in the poly C-directed reaction and is also the normal substrate *in vivo*. Addition of 4×10^{-5} *M* GTP to reactions containing 6×10^{-4} *M* ITP and 6×10^{-4} *M* CTP caused a pronounced lengthening of the lag phase and a lowered rate of synthesis of rIC. Under these conditions ^{3}H-GTP was incorporated into the polymer in positions adjacent to CMP residues.

FUTURE PLANS

We plan to continue our studies on the synthesis and structure of rIC copolymer. In particular the sedimentation coefficient, buoyant density and T_m will be determined and compared with that of the rI:rC homopolymer synthesized in the poly C-directed reaction. The effect of GTP on the reaction will be further investigated to determine whether GTP affects primarily the initiation process, or whether both initiation and polymerization are equally inhibited.

CHEMICAL AND OPTICAL PROPERTIES OF NUCLEIC ACIDS AND PROTEINS

(M. Maestre, K. Sieux, C. Hurlbut, E. Hong)

Our four main lines of research involve the study of nucleic acids, of the nucleic acid interactions with protein, and of protein structure, as follows:

A. Electrical-Optical Properties of Viruses

This research involves an intensive study of the T-even bacteriophages with emphasis on the T2 phage and the T4 phage and the amber mutants T4 (*am* N 58) which involve changes on the protein coat. The measurements will give the rotatory diffusion coefficients; the specific intrinsic birefringence and form birefringence; and the induced and intrinsic dipole strengths of the virus and its protein coat or ghost. These parameters, which are related to the general structure of the virus, will give insight into the forces involved in the interactions of the virus with its host cell and the self assembly *in vitro* of the component parts of the virus (head, tail, tail plate, and tail fibers). A manuscript entitled "Transient Electric Birefringence Studies on T2 Bacteriophage and its T2 Ghost", was submitted in May, 1967 to the *Journal of Molecular Biology*. Further work is being carried out on the amber mutants of T4 phage which lack the tail fibers, by a graduate student in biophysics, Mr. Charles Hurlbut.

B. Study of the Optical Activity of Various Types of Phages Both in Their Normal State and a Substituted State Where Nucleic Acid Analogues, Such as 5-Bromodeoxyuridine, 5-Iodeoxyuridine and 5-Fluorodeoxyuridine, are Incorporated.

The alteration of the structure of the nucleic acid in the internal state as compared to normal nucleic acid of the virus has given an insight on the distortions involved in the packing of the DNA molecule in the head of the phage. Very large changes in the rotational strength at 290 and 260 mμ in the ORD spectra occur in both the intact virus and on the isolated DNA of T2 phage. The structure of the nucleic acid is thus being altered by the presence

of the bromine and iodine atoms associated with the uracil. This alteration was compared with that seen in nucleic acids which had been altered by placement in very high salt concentration at neutral pH. Both show qualitative similarities in the distorted state ORD spectra. A probable explanation is that in both cases the stacking geometries of the bases are altered, with a consequent alteration of the optical activity of the molecule. In the viruses it is suspected that the alteration of the molecule is due to the extreme tightness of packing. Subsequent ORD measurements of density mutants of lambda phage, λb_2, $\lambda b_2 b_5$ which lack 12% and 24% respectively of their nucleic acid complement, showed results that agreed qualitatively with assumed geometrical distortion of the bases in the internal nucleic acids.

C. Protein -- Nucleic Acid Interactions
(in collaboration with Dr. J. S. Krakow)

The optical activity of the nucleic acid is used to study any possible changes in structure as it interacts with proteins such as polymerases. Interactions of DNA-dependent RNA polymerase with synthetic polymers such as alternating deoxy-AT copolymer and poly U are being observed by ORD. Further work is to be done on the other types of interactions of nucleic acids with proteins such as the internal proteins found in the head of T2 and T4 bacteriophages.

D. The Structure of Spinach Ferredoxin
(in collaboration with Dr. H. Matsubara)

The ORD spectra of spinach ferredoxin were measured in the range from 530 mμ to 195 mμ. By the use of the recently reported curves of different α-helical, β-structure and random coil content polypeptides (Greenfield, Davidson, and Fasman *Biochemistry*, *6*:1630 1967), the following values were obtained for the spinach ferredoxin molecule: the α-helical content is approximately of 20 to 26% of the molecule and the β-structure is from 40 to 44% of the molecule. The values for the α-helical content agree quite well with values computed by the method of Periti, Quagliarotti and Liquori (*J. Mol. Biol.*, *24*:313 (1967)).

FUTURE PLANS

1) In the future six months, we intend to study many of the assembly mutants, *i.e.*, imperfect head, tails, and tail fibers for their electro-optic properties.

2) Further work will be done to determine the quantitative relationship between the degree of incorporation of base analogues and the optical activity of the DNA molecule, both in the internal state in the virus, and free in solution.

3) Measurements of the optical activity of ferredoxins from other sources will be made.

SEQUENTIAL STUDY OF SPINACH FERREDOXIN: CONFIRMATION

(H. Matsubara and D. Ouye)

After completion of the sequence of spinach ferredoxin, a paper appeared on alfalfa ferredoxin (Keresztes and Margoliash, *J. Biol. Chem.*, *241*, 5955 1966) which suggested that the two ferredoxins had similar functional properties and chemical compositions. However, it was reported that alfalfa ferredoxin contained 6 cysteine residues — and 101-102 amino acid residues. Recently, at the *7th International Congress of Biochemistry* held in Tokyo, Japan, the authors reported that alfalfa ferredoxin contained 5 cysteines and 97 amino acid residues.

SEQUENTIAL STUDY OF CHROMATIUM FERREDOXIN

(H. Matsubara, R. M. Sasaki, and D. Ouye)

As the first step in the sequential study, the molecular weight and the amino acid composition of *Chromatium* ferredoxin were determined. The gel filtration method gave a molecular weight of about 10,000 and the amino-terminal analysis gave also a very close value, 12,210. On this basis the amino acid composition was determined as follows:

$Lys_2His_2Arg_2Asp_9Thr_6Ser_4Glu_{16}Pro_5Gly_5Ala_4Val_6Ile_7Leu_3Tyr_3Phe_0$
$Trp_0Met_1Cys_9$, total 84. The molecular weight corresponded to 9,250 excluding iron and labile sulfur.

The purification procedure gave a low recovery of ferredoxin but crystalline material was easily obtained. The purified material resembled the clostridial type of ferredoxin in its absorption spectrum, which had a flat peak between 278 mμ and 282 mμ, a shoulder at 310 mμ and a broad peak at around 385 mμ.

Tryptophan was absent and one methionine residue was present. Although the analysis showed 0.31 mole phenylalanine per mole of protein, we assumed that no phenylalanine was present.

The compositions of various ferredoxins are shown in Table 1 which includes a ferredoxin from a unicellular green alga, *Scenedesmus*. The ferredoxins may be placed in three groups: type i, from green plants and green algae; type ii, from photosynthetic bacteria; type iii, from nonphotosynthetic bacteria.

It is interesting that *Chromatium* ferredoxin shows some characteristics intermediate between those of ferredoxins of types i and iii. The total content of basic amino acids is close to that of the plant type ferredoxins although *Chromatium* ferredoxin is lower in lysine. The glutamic acid plus glutamine content is higher than that of aspartic acid plus asparagine. There is no tryptophan, and the content of half-cystine is high; in these respects, it resembles the bacterial type. Its content of isoleucine is higher than that of leucine. The amino-terminal residue was identified as alanine, 8.19 mole/10^5 g, without any correction for the losses, 10—15% during the process of identification.

The fact that methionine was found in *Chromatium* and *Scenedesmus* ferredoxins may be contrasted with the finding that the ferredoxins of Swiss chard (Matsubara, unpublished), of taro (Rao and Mower, personal communication, 1967) and of nonphotosynthetic bacteria lack methionine.

TABLE 1

COMPARISON OF AMINO ACID COMPOSITIONS OF FERREDOXINS

	Type i			Type ii	Type iii		
	Spinach (a)	Alfalfa (b)	*Scene-desmus* (c)	*Chroma-tium* (d)	*Cl. past.* (e)	*Cl. buty.* (e)	*Micrococcus aerogenes* (f)
Lysine	4	5	4	2	1	0	1
Histidine	1	2	1	2	0	0	0
Arginine	1	1	1	2	0	0	0
Aspartic Acid + Asparagine	13	10	12	9	8	9	8-9
Threonine	8	6	10	6	1	3	0
Serine	7	8	8	4	5	3	5
Glutamic Acid + Glutamine	13	17	10	16	4	5	4
Proline	4	3	4	5	3	3	5
Glycine	6	7-8	7	5	4	5	4-5
Alanine	9	10	10	4	8	7	8
Valine	7	9	5	6	6	6	4
Methionine	0	0	1	1	0	0	0
Isoleucine	4	4	3	7	5	4	6
Leucine	8	6	7	3	0	0	0
Tyrosine	4	4	4	3	1	0	2
Phenylalanine	2	2	3	0	1	2	0
Tryptophan	1	1	0	0	0	0	0
Half-cystine	5	6	6-7	9	8	8	8
Total	97	101-102	96-97	84	55	55	55-57
Iron	2	2	2	(4)	7	7	
Labile sulfur	2	2	2	(4)	7	7	

(a) Matsubara et al. (1967)
(b) Keresztes-Nagy and Margoliash (1966)
(c) Matsubara, in preparation
(d) Present communication
(e) Benson et al. (1966)
(f) Tsunoda et al. (1967)

PURIFICATION AND CHARACTERIZATION OF *Scenedesmus* FERREDOXIN

(H. Matsubara)

The unicellar organism, *Scenedesmus*, was chosen for the sequential study of its ferredoxin. A very simple purification procedure was developed. It includes the breakage of cells by glass beads with a blendor, DEAE-chromatography, ammonium sulfate fractionation, sephadex column diromatography hydroxylapatite column chromatography, and crystallization. The material was shown to be pure by disc electrophoresis and centrifugation.

The absorption spectrum was that of a typical plant type. It had 2 labile sulfurs and 2 iron atoms per molecule. The activity of *Scenedesmus* ferredoxin was very similar to those of spinach and Swiss chard. The amino acid composition was determined as shown in Table 1.

SEQUENTIAL STUDY OF THERMOLYSIN

(H. Matsubara and D. Ouye)

The amino-terminal sequence was determined to be Ile-Thr-Gly-Thr by Edman procedure and the carboxyl-terminal Tyr.

FUTURE PLANS

The sequential studies of *Chromatium* or *Scenedesmus* ferredoxins will be continued. Some work on chemical modifications will be conducted with *Scenedesmus* ferredoxin.

EVOLUTION OF OXYGENASE OF AROMATIC COMPOUNDS IN PSEUDOMONADS

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

Pseudomonads, soil bacteria, are highly adaptable organisms, and in soil they convert various organic compounds to simple inorganic compounds, (den Dooren de Jong, 1926). *Pseudomonas putida* M-6 (ATCC No. 17428) is a soil isolate (K. Hosokawa, 1959) capable of growing on p-hydroxybenzoic, salicylic, benzoic, or anthranilic acids as the sole source of carbon and energy. A total of 13 specific enzymes are involved. These enzymes are all inducibly

synthesized in response to aromatic compounds. The close similarities of the enzymes in analogous reaction steps 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 was undertaken to investigate this, because the two enzymes are similar in nature. We have prepared p-hydroxybenzoate hydroxylase in crystalline form starting from 3 kilograms of bacterial paste. The enzyme has FAD as a prosthetic group and requires NADPH and molecular oxygen besides p-hydroxybenzoic acid which is converted to protocatechuic acid. Purification of salicylate hydroxylase is in progress. It is also a FAD* enzyme having properties very similar to p-hydroxybenzoate hydroxylase except that it has a requirement for NADH* instead of NADPH*. P-hydroxybenzoate hydroxylase consists of at least 4 subunits of different electrophoretic mobilities. The substructure of salicylate hydroxylase is being investigated; it is likely that it also consists of subunits. If p-hydroxybenzoate and salicylate hydroxylases both have the same evolutionary origin, a common subcomponent might be retained. Reconstitution of hybrid enzyme by exchanging subunits between two hydroxylases will be carried out to test this possibility.

We are preparing to investigate genetic control mechanisms of oxygenases including hydroxylases. Mutants lacking p-hydroxybenzoate, salicylate, including benzoate and anthranilate hydroxylases, were obtained by microcolony isolation technique combined with the penicillin screening method. Mutants lacking protocatechuate- and catechol-oxygenase are being examined.

---

*FAD = Flavin adenine dinucleotide, NADH = Nictinamide adenine dinucleotide; NADPH = Nictinamide adenine dinucleotide phosphate.

FUTURE PLANS

1. Comparative biochemistry of hydroxylases.

2. Evolutionary aspects of genetic control mechanism of oxygenases including hydroxylases.

The mechanism of hydroxylation is being investigated in collaboration with Dr. Barrie Hesp (Laboratory of Chemical Biodynamics).

FUNCTION AND STRUCTURE OF BACTERIAL RIBOSOMES

(K. Hosokawa, M. A. Q. Siddiqui, S. Goolsby)

A specific fraction of structural protein separates termed "split proteins" (SP30 and SP50) when ribosomes of *E. coli* (30S and 50S = 1.6) are subjected to CsCl equilibrium density gradient centrifugation and the intact ribonucleoprotein particles form a band of density 1.65 called "core particles" (23S and 40S). Units with the original sedimentation constant are formed which have their original function in terms of polyuridylic-acid directed phenylalanine incorporation (PUPI) by mixing the split proteins with core particles. Electrophoresis of ribosomal proteins in the presence of 6-M urea showed that 30S and 50S ribosomes contain respectively 13 and 17 different proteins.

We started further fractionation of split proteins from 30S ribosomes. Fractionation of SP30 was carried out by DEAE-cellulose column chromatography in 6-M urea. The fraction which passed through the column was termed *basic protein fraction* (SP30B) and the fraction adsorbed and eluted by salt was called *acidic protein fraction* (SP30A). The former is indispensable in peptide synthesis and the latter is dispensable. The basic protein fraction (SP30B) was further fractionated into 5 subcomponent proteins by cellulose cation exchange column chromatography in 6-M urea. Purity was estimated at over 95% as judged by polyacrylamide gel electrophoresis. Five kinds of 30S ribosome particles, each of which lacked one of the 5 components of SP30B, were produced *in vitro* by mixing 23S core particles, plus SP30A, plus components of SP30B. PUPI in the presence

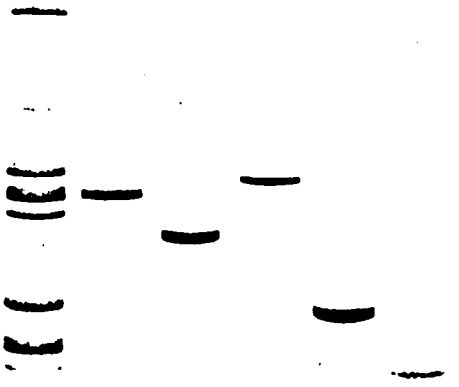

Figure 1. Acrylamide gel electrophoresis pattern of five basic proteins isolated from split proteins of 30S ribosome. From left to right, 30S ribosome proteins followed by five basic proteins of SP30B.

of these particles was measured and compared with that obtained with completely reconstituted 30S ribosomes. It was found that three protein sub-units were indispensable and two were dispensable. We are preparing the five components in a large scale. Characterization of these components, especially the three indispensable functional protein subunits, is being undertaken. Physicochemical properties and amino acid sequence will be measured.

FUTURE PLANS

We plan to develop a method to split core particles from antibiotic-resistant organisms into RNA and protein and to reconstitute core particles from them for further study of codon-anticodon recognition. Mutational modifications of ribosomal structure, which cause misreading of codons, will be used to investigate this. Hybridization of ribosomes from components of different organisms will be studied.

Pseudomonas putida and *Pseudomonas acidovorans* can be distinguished from each other by biochemical characteristics, and are two representative species of *Pseudomonas*. These will be used as sources of ribosomes for hybridization experiments in which we shall see how many components of the ribosome are interchangeable between the two species. The ribosome is an organelle that is found in all organisms and is suitable for comparative studies as a measure of evolutionary divergence. Ribosomes of thermophilic and psychrophilic organisms will be examined to explore the influence of temperature on a living system using the technique of reconstitution of hybrid ribosomes. Isolation of RNase negative mutants of these bacteria will be attempted, because RNase attacks ribosomal RNA during the manipulations necessary for hybridization.

FORMATION OF BACTERIOPHAGE T4

(J. Hosoda and E. Mathews)

I. DNA SYNTHESIS BY LIGASE-DEFECTIVE MUTANTS OF T4

Replication of T4 DNA is characterized by its high rate of recombination. The process involves enzymatic breakage and joining of preformed polynucleotide strands. Richardson has studied a T4 induced enzyme, polynucleotide ligase, which catalyzes the repair of single-strand breaks by the formation of phosphodiester bonds and has located the structural gene for the enzyme. It has been found that DNA synthesis induced by amber mutants in the same gene starts at the normal time but quickly stops and the newly synthesized DNA is slowly degraded to TCA-soluble materials (Hosoda, *BBRC 27*:294, 1967). We studied the sizes of newly synthesized DNA and parental phage DNA, after infection of ligase-defective amber mutants in restricted condition, to investigate the possible role of polynucleotide ligase in phage DNA synthesis and recombination.

A considerable portion of new DNA is associated with a large structure which sediments faster than mature DNA, although this new DNA consists of single strands much shorter than mature DNA when examined in alkali gradients. The new DNA separated into two peaks in alkali sucrose gradients, one broad peak which sedimented as fast as T7 DNA (1/5 the size of T4 DNA) and a sharper peak which sedimented much more slowly, apparently representing fairly homogeneous short segments.

These two new DNAs become shorter upon further incubation. Replicating DNA disappears in neutral gradients. The single-strand peak in alkali gradients, which sedimented faster than mature DNA, became shorter.

The parental DNA became shorter during the infection: the average molecular size of single strands at the time when DNA synthesis had almost stopped was about 1/2 of mature DNA size.

II. ASSEMBLY OF T4 HEADS FROM PROTEIN SUBUNITS AND DNA

It is assumed that the presence of DNA as a core is necessary for coat protein subunits to assemble and to make heads of T4 phage. Polynucleotide-ligase defective mutants do not accumulate DNA, but can synthesize head protein subunits. Integration of subunits into head-like structures by these mutants has been investigated. The results so far indicate a strong correlation between availability of large-size DNA (possibly by replicating form), and integration of head proteins. The ligase mutation seemed to inhibit the integration of head proteins strongly but did not stop it completely. The rate of integration was greater in the earlier period than later, which indicated a correlation between the size of DNA and head integration.

FUTURE PLANS

1. DNA synthesis by ligase defective mutants of T4 phage

If DNA synthesis by T4 phage is mediated solely by action of DNA polymerase which adds mononucleotide-5'-phosphate to the 3-hydroxyl end of polynucleotide chains, and DNA synthesis starts at one end of double stranded DNA, the direct product could be a continuous polynucleotide chain for one strand but a collection of short-chained segments for the other strand. If this is the case, a polynucleotide-ligase-type enzyme is necessary to connect the short segments. The appearance of two groups of single strands in DNA made by a ligase-defective mutant is of interest in this regard. Our future work will be to investigate if these two groups of single strands are derived from the two different stands of DNA molecules.

2. Head assembly

The problem of head assembly will be continued by studying the products from head subunits in the absence of normal DNA.

REGULATION OF NUCLEOSIDE METABOLISM IN *E. coli*

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

E. coli can utilize nucleosides for the synthesis of nucleic acids, and can also degrade the sugar groups of nucleosides for the production of energy. The pathways of nucleoside degradation involve the formation and cleavage of pentose phosphates by enzymes which are normally constitutive in *E. coli*. However, most of these enzymes can be optimally induced during growth of *E. coli* in the presence of appropriate nucleosides. We are examining the physiological and genetic regulation of these enzymes, and are attempting to characterize more fully some of the individual enzymatic steps.

A. Enzyme Induction Patterns

The enzymes we have examined can be grouped into several distinct classes on the basis of their inducibility by specific nucleosides: (1) One group, composed of thymidine phosphorylase (TP) and deoxyribose phosphate aldolase (DPA) is fully induced only during growth with deoxyribonucleosides, but not with ribosyl compounds. Under a wide variety of inducing and growth conditions, TP and DPA are induced and repressed in parallel, suggesting a coordinate genetic control for these two enzymes. We have also obtained several mutants which are jointly altered in the basal levels of these two enzymes, and which may contain regulatory gene mutations in this operon. (2) A second group of enzymes, typified by purine nucleoside phosphorylase (PNP) and phosphodeoxyribomutase (PDM) is maximally induced with either deoxyribonucleosides, or with purine ribonucleosides. We do not yet know whether these enzymes are coordinately regulated. (3) In contrast to the above groups, several enzymes are induced chiefly on the basis of base specificity. *E.g.*, adenosine deaminase is inducible by adenine and hypoxanthine derivatives, and uridine phosphorylase is induced solely by cytidine (but not by uridine). From these patterns, it is evident that the pathways of nucleoside degradation involve the interaction of a number of different operons.

B. Isolation and Properties of Mutant Strains

In order to analyze the physiological role of these enzymes, we have examined the behavior of mutants of *E. coli* B which lack the individual enzyme activities. Since these enzymes participate in reactions which lead to the utilization of nucleosides for energy, the mutant strains have been selected by their ability to utilize specific nucleosides as sole carbon sources. TP⁻ mutants are unable to grow on thymidine, but can grow on purine nucleosides; mutants defective for PNP show the converse behavior. Strains lacking PDM or DPA do not readily grow on deoxyribonucleosides. Representative mutants of these types have been isolated and characterized on the basis of their growth patterns, enzymatic profiles, and inducibility of non-mutated enzymes. Some of their properties are tabulated below:

PROPERTY	*E. coli* STRAIN				
	B	TP⁻	PNP⁻	PDM⁻	DPA⁻
Growth on the following carbon sources:					
Thymidine	+	–	+	–	–
Uridine	+	+	+	–	+
Deoxyinosine	+	+	–	–	–
Inosine	+	+	–	–	+
Cytidine	+	+	+	sl.	+
Glucose	+	+	+	+	+
Induction by:					
Deoxyinosine	+	+	–	–	+
Thymidine	+	–	+	–	+
Inhibition by deoxyribonucleosides	–	–	–	–	+

Strains which are defective for TP, PNP, or DPA are induced for non-mutated enzymes when grown with inducing nucleosides, but PDM⁻ mutants cannot be induced under comparable conditions. Since PDM⁻ mutants can generate pentose-1-phosphates, but not pentose-5-phosphates from nucleosides, this finding strongly indicates that the pentose-5-phosphates serve as the proximal intracellular inducers for TP, DPA, PNP, and possibly PDM.

The mutase probably also participates in the metabolism of ribose phosphates, since (a) this enzyme is fully induced by purine ribonucleosides, and (b) since PDM⁻ mutants are unable to grow on most ribonucleosides as sole sources of carbon. These mutants show limited growth in the presence of cytidine. This fact, and the unusual inducing properties of cytidine (which acts like a weak deoxyribosyl inducer) suggest that *E. coli* can convert the ribose portion of cytidine to deoxyribose-5-phosphate by reactions which are independent of PDM. We have examined extracts of PDM⁻ strains for an enzyme activity which cleaves dCMP or dTMP at the glycosyl bond, with the release of deoxyribose-5-phosphate, but none has been found.

The induction of PDM in *E. coli* B is not accompanied by comparable changes in the activity of phosphoglucomutase, and normal levels of phosphoglucomutase are present in strains devoid of PDM activity. Therefore, these two enzymes appear to be distinct protein entities in *E. coli*, although the possibility exists that they may differ only in the association of common protein subunits.

The growth of DPA⁻ mutants, both thymine-independent and thymine-requiring, is inhibited by deoxyribonucleosides. This inhibition appears to be a result of the accumulation of deoxyribose-5-phosphate or its metabolites, and is completely reversed by supplementation with ribonucleosides. These findings indicate that *E. coli* can dispose of deoxyribose-5-phosphate by reactions other than DPA, possibly by the transketolase reaction, in which deoxyribose-5-phosphate serves as a ketol acceptor (Allende and Racker, *Biochim. Biophys. Acta,*). The properties of DPA⁻ strains are very similar to

those of the "deoxyribonucleoside sensitive" (d^S) strains, recently described by Lomax and Greenberg (*Bact. Proceedings*, 1967, p. 56). We are attempting to determine whether the DPA gene is equivalent to the d^S gene, or whether these are closely linked but distinguishable.

C. Future Plans

1. Examination of other enzymes for induction during growth with nucleosides will be made. Of particular interest are those involved in the metabolism of ribose-5-phosphate, such as transketolase, phosphoriboisomerase, and ATP-PRPP pyrophosphorylase. The regulation of these enzymes is of considerable interest, since they represent a group of enzymes at a metabolic branch point.

2. Attempts to find a gratuitous inducer for these degradative pathways will be made.

3. Efforts in the purification and characterization of phosphodeoxyribomutase will be continued.

4. The loci for which mutants are available will be mapped genetically. We shall attempt to construct recipient strains and to carry out initial conjugational analysis. There is reason to believe that the TP and DPA genes are located in the leu-thr region of the chromosome.

BACTERIOPHAGE INTERNAL PROTEINS

(H. O. Kammen and M. Strand)

The internal proteins of the T-even bacteriophages constitute a small, but immunologically distinct fraction of the viral protein, which is injected into the host cell during infection. The functions of the internal proteins are not known, although it has been suggested that they are involved in the condensation of viral DNA prior to its packaging in the phage head. We have undertaken to reinvestigate the chemical nature and role of these internal proteins.

Several attempts to prepare T2 and T4 internal proteins by a new procedure (Bachrach and Friedmann, *BBRC*, *26*:596, 1967) were repeatedly unreproducible. Milligram amounts of T2 and T4 internal proteins have been obtained by older methods from osmotically disrupted phages, and have been used for the preparation of the respective rabbit antisera. When tested by the Ouchterlony technique, no serological cross-reactions were obtained between T2 and T4 internal proteins.

When native T4 or T7 DNA is mixed with internal proteins at low ionic strength, it is retained by Millipore membranes, in contrast to unbound DNA. By employing ^{3}H-T7 DNA, the radioactivity retained on the membrane at saturating concentrations of DNA is a rough indication of the amounts of internal protein bound.

Polyacrylamide gel electrophoresis of the purified internal proteins of T4 has disclosed that they are heterogeneous fractions, containing two major protein constituents, and several trace components.

The immediate objectives of this project are as follows:

1. Resolution of the individual components of the internal protein fractions and determination of their amino acid compositions.

2. Screening of viral mutants to determine which cistrons may control the synthesis of internal proteins. To this end, extracts from non-permissive infections will be assayed for internal proteins antigens by a highly sensitive microcomplement fixation technique. Miss Strand recently spent several days in Dr. Allan Wilson's laboratory learning the method. If a cistron is found for which an internal protein is a gene product, we will examine the type of viral structures which accumulate during non-permissive infection. This analysis may give clues to the function of internal proteins.

3. We are also planning experiments to test whether the injection of internal proteins during infection conveys any genetic specificity, and other experiments to determine whether the internal proteins are bound to the viral DNA in a random or non-random manner.

DETERMINATION OF BASE SEQUENCES IN TOBACCO MOSAIC VIRUS RNA (TMV-RNA)

(S. Mandeles, F. J. Fearney, R. Anderson)

Work has continued on the development of a stable label on the 5'-linked terminus of TMV-RNA. It has been found that reduction of the bis-semi-carbazone of TMV-RNA with $NaBH_4$ produces a label of increased stability. This increase in stability is particularly noticeable during chain length separation on DEAE-Sephadex. Unfortunately, the reduced bis-semi-carbazone label is not stable to treatment with charcoal so that an alternative method for recovering labelled fragments from high salt solution is being sought.

The use of ^{32}P as a label for the 3'-linked terminus is also currently under investigation. Pancreatic RNase and T_1 RNase digests have been made of ^{32}P-TMV-RNA. Chromatographic analyses of the digests have shown indications of a contaminating nuclease and phosphotase. Experiments are under way to eliminate these contaminations.

FUTURE PLANS

Work is planned along three lines:

1. Continuation of base sequence experiments with ^{32}P and ^{14}C labels for the 3'-OH linked and 5'-OH linked ends (respectively) of TMV-RNA.
2. Isolation, purification, and characterization of oligomers of unique sequences from T_1 RNase digests of TMV-RNA.
3. Use of oligomers of unique sequences to map TMV-RNA.

ACTION OF VARIOUS MUTAGENS ON TMV AND TMV-RNA

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

The decrease in infectivity upon reaction of TMV-RNA with methyl-nitro-nitrosoguanidine (MNNG) differs depending on the solvent system, ionic strength, and temperature. In order to attempt a correlation of chemical events, mutagenesis, and infectivity, all of the variables were studied.

The first observations were 1) an increase in ionic strength decreased the infectivity loss (Table I); 2) infectivity was lost very slowly in 67% dimethyl formamide (DMF), less slowly in formamide (FA) and rapidly in H_2O at 20°C (Table I), and 3) an increase in temperature from 20°C to 37°C slowed the infectivity loss in H_2O markedly (Table II). All of these observations could be explained in terms of changes in stacking of the bases. That is, the more base interactions, the greater the infectivity loss. This would lead to the hypothesis that MNNG inactivation is favored, when the bases are stacked.

It was previously reported that the mutagenicity was low for MNNG acting on TMV-RNA but high if the RNA was still in the virus where presumbably there are no base interactions. It now appears that in 67% dimethyl formamide (DMF) there is little increase in mutation rate, even though the infectivity and chemical reactions differ from the reaction in H_2O. This paradox is further complicated by the fact that reaction in formamide (FA) leads to a marked increase in mutation, while the inactivation in FA lies between that in H_2O and DMF, and base changes are minimal.

An illustration of base changes observed with different solvents is shown in Table III. The data presented are for S-RNA since this RNA has a high GC content. Similar analytical results have been obtained with TMV-RNA and yeast RNA.

The data show that neither the G product nor the C product are produced in analytically detectable amounts when the reaction is carried out in FA. Only in reactions carried out in DMF is the C product found as well as the G product. The greatest amount of the G product is found when MNNG reacts with RNA in aqueous solutions at 37°C. The data also show that the chemical changes are favored by higher temperatures, whereas the inactivation rate (Table II) is favored by lower temperatures. It is at the lower temperature that

mutation has been studied so far. Since the magnitude and nature of the chemical studies show no evident relationship to the biological studies, further work on the effect of temperature and solvents is planned.

TABLE I

INFECTIVITY OF TMV-RNA REACTED WITH MNNG
IN VARIOUS SOLVENTS AT 20°C

Solvent [a]	buffer	Time 15 minutes	60 minutes
		% Remaining Inactivity	
Formamide	none	2.5	0.1
	PO_4	30	4
67% Dimethyl Formamide	none	15	0.4
	PO_4 [b]	69	39
H_2O	none	0.4	0.08
	PO_4	2.3	0.07

a) 660δ MNNG/mg RNA/mℓ solvent

b) 0.04 M pH 7 phosphate, 0.05 M pH 4.5 acetate, or 0.05 M NaCl all produced similar effects when added to 67% DMF

TABLE II

EFFECT OF TEMPERATURE ON INFECTIVITY OF TMV-RNA REACTED WITH MNNG

Reaction Conditions [a]			% Remaining Infectivity		
	mixed	held	30 seconds	10 minutes	30 minutes
H_2O	20°C	20°C	49,(70)	0.06,(0.4)	
	20°C	37°C	82	18	(4)
	37°C	37°C	185,(79)	220,(147)	(0), (0.4)
67% DMF	20°C	20°C	(40)	15	0.7
	20°C	37°C	(150)	31	1.3
	37°C	37°C	(80)	31	3.7

a) 3 mg MNNG/mg RNA without buffer

Figures in parentheses are single assays

TABLE III

BASE RATIOS OF S-RNA REACTED WITH MNNG IN VARIOUS SOLVENTS

Reaction Conditions (a)	Method of Hydrolysis	Base Ratios A	G	U	G	G Product	C Product
Formamide 37°C	HCl	1.0	1.63	0.70	1.34		0
	SV-DE	1.0	1.72	0.87	1.36	(?)	
67% Dimethyl Formamide 37°C	HCl (b)	1.0	1.52	0.80	1.19		0.18
	SV-DE (b)	1.0	1.44	0.83	1.27	0.29	
H_2O 37°C	HCl (b)	1.0	1.04	0.79	1.31	0.79	
	SV-DE (c)	1.0	1.15	0.83	1.33	0.65	
H_2O R.T.	HCl	1.0	1.33	0.75	1.36	0.21	0
	SV-DE	1.0	1.13	0.90	1.41	0.41	
H_2O + TMV Protein 37°C	HCl	1.0	1.34	0.81	1.41	0.45	0
Untreated	HCl	1.0	1.65	0.80	1.30		

SV-DE = SNAKE VENOM DIESTERASE

a) All reactions were without buffers for 2–3 days with 3mg MNNG/mg RNA

b) AV. of 3 experiments

c) AV. of 2 experiments

PERSONNEL

Participating Faculty

Professor Thomas H. Jukes — Principal Investigator; Professor-in-Residence
Professors Daniel I. Arnon; Heinz Fraenkel-Conrat; Hardin B. Jones; Ignacio Tinoco; Visiting Professor — Henry Borsook

Non-Faculty Professional Research

Beatrice Fraenkel-Conrat; Junko Hosoda; Keiichi Hosokawa, Joseph S. Krakow; Harold O. Kammen; Marcos F. Maestre; Stanley Mandeles; Hiroshi Matsubara; Mituru Takanami; Hiroshi Yoshikawa

Graduate Students — Martin Haas; Myra Karstadt; Helen Mayoh

Post Graduate Research Chemist — M. A. Q. Siddiqui

Post Graduate Research Chemist (Non Student) — Mette Strand

Clerical — Roselyn Ferreira; Constance Reid; Clarice Garrison

Technicians

Richard Anderson; Estelle Cook; Frank Fearney; Earl Fronk; Barbara Jansen; Elaine Mathews; Dorinne Ouye; Kimmie Sieux; Ruth Siegel; Ella Turner; Margaret Larsen; Mariko Kamio; Sara Goolsby; Judith Levitt; Eppie Sheng; Richard Sasaki; Jean Jansen

Laboratory Helpers

Cynthia Schieberl; William Clark; Lester Franklin; Lonnie Johnston; Elaine Newton

PUBLICATIONS:

Matsubara, Hiroshi. Some properties of thermolysin. *Molecular Mechanisms of Temperature Adaptation.* American Association for the Advancement of Science (1967).

Singer, B., and H. Fraenkel-Conrat. Chemical modifications of Viral RNA, VI. The action of N-methyl-N-nitro-N-nitrosoguanidine. *Proc. Natl. Acad. Sci. U.S., 58*:234 (1967).

Okamoto, Toshio. Factors from *Bacillus stearothermophilus* that inhibit the binding of amino acids to transfer RNA. *Biochim. Biophys. Acta, 138*:198–200 (1967).

Kammen, Harold O., and Mette Strand. Thymine metabolism in *Escherichia coli*. II. Altered uptake of thymine after bacteriophage infection. *J. Biol. Chem., 242*:1854 (1967).

Mandeles, S. Base sequence at the 5'-linked terminus of TMV ribonucleic acid. *J. Biol. Chem., 242*:3103 (1967).

Yoshikawa, H. The initiation of DNA replication in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U.S., 58*:312 (1967).

Jukes, Thomas H. Indications of an evolutionary pathway for the amino acid code. *Biochem. Biophys. Res. Commun., 27*:527 (1967).

Sasaki, R., and H. Matsubara. Molecular weight and amino acid compositions of *Chromatium* ferredoxin. *Biochem. Biophys. Res. Commun., 28*:467 (1967).

Hosoda, J. A mutant of bacteriophage T4 defective in α-glucosyl transferase. *Biochem. Biophys. Res. Commun., 27*:294 (1967).