lagen. However, in both cases the trypsin degraded the substrate less than one-third as rapidly as equivalent amounts of collagenase or cell homogenate under the conditions of these experiments.

These data indicate the existence in bone cells of an enzyme activity capable of breaking down the collagen of bone matrix at least to ultrafilterable fragments. The physiological implications of these findings are of obvious interest and raise questions concerning the site of synthesis and storage and mode of release of this activity in skeletal tissue. In preliminary experiments in which intact bone fragments were incubated in vitro for prolonged periods in nonradioactive media after labeling of their collagen with radioactive proline, the collagen activity remained unchanged for at least 48 hours (Fig. 1). Apparently, the cells in areas of bone where matrix synthesis is in progress either do not contain or do not release this enzyme activity.

In addition, more vigorous disruption of isolated cells from bone increases the relative activity of the homogenate suggesting that the enzyme may be contained in subcellular organelles, perhaps lysosomes, and is released or activated by mechanisms not yet understood. The results of preliminary experiments indicating that all the activity is in the particulate fraction of the cell homogenate support this view.

JOHN F. WOODS
GEORGE NICHOLS, JR.
Department of Medicine, Harvard Medical School, Boston, Massachusetts

References and Notes

1. I. Mandl, *Advan. Enzymol.* **23**, 164 (1961); D. E. Oken and R. J. Boncek, *Proc. Soc. Exptl. Biol. Med.* **96**, 367 (1951).
2. J. Gross and C. M. Lapiere, *Proc. Natl. Acad. Sci. U.S.* **48**, 1014 (1962).
3. B. Flanagan and G. Nichols, Jr., *J. Biol. Chem.* **237**, 3686 (1963).
4. ———, *Federation Proc.* **22**, 553 (1963).
5. ———, *Endocrinology*, in press.
6. Supported by grant A-854 (c7) from the National Institute of Arthritis and Metabolic Diseases and by grants from the Proctor and Milton funds of Harvard University.

20 August 1963

Transcription in vivo of DNA from Bacteriophage SP8

Abstract. *The DNA of bacteriophage SP8, when denatured, yields two components differing in buoyant density in cesium chloride gradients and separable by chromatography on a column of methylated bovine serum albumin and kieselguhr. The denser of the two strands (H) contains more pyrimidines and fewer purines than the lighter (L) strand. Only the H strand forms hybrids with the RNA synthesized by the infected host. The L strand is capable of annealing with complementary RNA synthesized in vitro with it as primer in reactions catalyzed by RNA polymerase. During the vegetative development of phage, host-specific messenger RNA is also synthesized.*

The properties of messenger RNA (mRNA) have been deduced from a variety of systems. Bautz has presented evidence that mRNA specific for bacteriophage may be single-stranded (*1*). Genetic evidence, obtained by Champe and Benzer (*2*) with the same coliphage system, suggests that the template for useful synthesis of RNA is one of the two complementary strands from bacteriophage DNA. However, experiments in vitro show that both strands of DNA are copied by the DNA-primed RNA polymerase (*3*). The availability of bacteriophages whose DNA's yield strands which can be fractionated and identified by their different buoyant densities has made it possible to test directly whether the RNA formed after phage infection is complementary to one or both of the strands of the bacteriophage DNA.

We have selected for study bacteriophage SP8 (*4*) which is virulent for *Bacillus subtilis* Marburg. This phage produces prompt and reproducible lysis of host suspensions grown in broth and synthetic media, and it yields clear plaques on solid media. By chemical analysis, the base composition of its DNA is similar to that of its host; the combined content of guanine and cytosine is 43 percent (*5*). When this DNA is heated (denatured), the separate strands show bands at distinctly different densities in the CsCl gradient. The separated strands can be fractionated by chromatography on a column containing methylated bovine serum albumin and kieselguhr by selective, discontinuous elution with saline-phosphate (*6*). The buoyant densities of this DNA in the native form and of its fractionated complementary strands, L and H, are 1.743, 1.755, and 1.764 g/ml (*5*). Both strands of DNA from phage SP8 can serve in vitro as templates for the RNA polymerase from *Escherichia coli*; from the incorporation of labeled ribonucleotides, it is apparent that the strand with the light buoyant density (the L strand) differs in base composition from the strand with heavy buoyant density (the H strand), but that they are complementary in their base composition (*7*). As in the case of bacteriophage α (*8*), the H strand is relatively rich in pyrimidines and the L strand is relatively rich in purines.

That RNA complementary to one of the strands of DNA from bacteriophage SP8 is synthesized in infected *Bacillus subtilis* was demonstrated by the principle of the DNA-RNA hybridization (*9*) which has been used for the isolation and identification of specific mRNA. In our case, the separation induced by denaturation and subsequent purification of the complementary strands permitted us to ascertain which of the strands served as a template for the synthesis of complementary RNA in vivo. The hybrid DNA-RNA molecules prepared by incubation of the DNA and RNA at 57°C (annealing) were separated by preparative CsCl density gradient centrifugation (*9*) and also by the use of an agar gel containing denatured DNA (*10*).

Labeled RNA isolated from SP8-infected *Bacillus subtilis* was first hybridized by annealing it with the H and with the L strands (SP8 DNA) previously separated chromatographically on the methylated bovine serum albumin column. After preparative centrifugation of the hybridization mixture in a CsCl density gradient, drops were collected from the bottom of the punctured centrifuge tube and the acid-precipitable fraction was assayed for radioactivity (after incubation either with or without ribonuclease). In Fig. 1 the extent of hybridization of the H and L strands of SP8 DNA with RNA from phage-infected *B. subtilis* is compared. Results obtained from samples treated with ribonuclease indicate clearly that only the RNA associated with the H strand has the resistance to ribonuclease which is expected of DNA-RNA hybrids (*11*). The amount of radioactivity in the fraction, resistant to ribonuclease and precipitable by acid, that is renatured with the H strand represents 10 to 15 percent of the labeled RNA added to the annealing mixture. On the other hand, the radioactivity associated with

the L strand is about 0.5 percent of that added to the annealing mixture; this could reflect contamination of the L strand in the annealing mixture by the H strand. Figure 1 shows a shoulder on the light-density side of the H strand–RNA hybrid which is not seen with a more highly purified preparation of H strand. No detectable hybridization of SP8-specific RNA could be observed with calf-thymus DNA or with either of the purified strands of bacteriophage α.

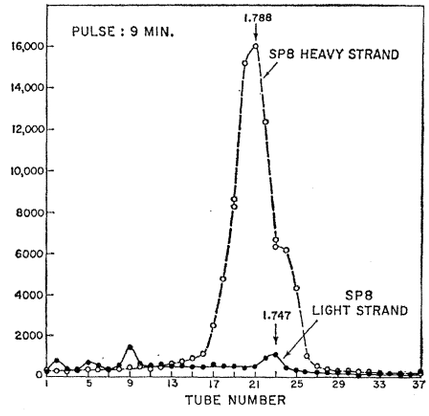

Fig. 1. Specificity of hybridization of RNA isolated from SP8-infected *B. subtilis* with the fractionated strands of SP8 DNA. Ribonuclease-resistant radioactivity. Ordinate: Radioactivity (count/min) of RNA labeled with H^3-uridine (after hybridization with fractionated phage DNA) of the CsCl density gradient fractions, precipitated with trichloroacetic acid after treatment with ribonuclease. Abscissa: CsCl fractions collected after preparative centrifugation. The hybridization mixtures, in 0.3*M* NaCl plus 0.03*M* sodium citrate, consisted of 32 μg of RNA labeled with H^3-uridine (6×10^5 count/min per microgram of material precipitable by cold trichloroacetic acid) and 10 μg each of the fractionated L and H strands of SP8 DNA in a total volume of 0.55 ml. To produce hybrids, the mixtures were incubated at 57°C for 10 hours (annealing) and cooled slowly to room temperature. Solid optical-grade CsCl and water were added to yield a density of 1.74 g/ml in a final volume of 3 ml. The samples were centrifuged at 33,000 rev/min for 3 days at 25°C in the SW 39 rotor of a Spinco model L ultracentrifuge. Fractions were obtained by piercing the lower end of the tube and collecting samples of 3 drops. After every 5 to 8 drops, the refractive index of one drop was measured. To each 3-drop sample was added 2 ml of water containing 10 μg of ribonuclease and 50 μg of calf-thymus DNA (Worthington) as carrier. After a 22-minute incubation at room temperature, the nucleic acids were precipitated with cold 20-percent trichloroacetic acid collected on HA Millipore filters, and were assayed for radioactivity by scintillation spectrometry (*9*). Densities of the peaks were calculated from the refractive indexes. RNA labeled with H^3-uridine was obtained from *B. subtilis* infected in the logarithmic phase (multiplicity of infection, 5) in a medium similar to that of Nomura *et al.* (*20*). After "pulse labeling" (3.5 to 12.5 minutes after infection), the bulk RNA was isolated with warm phenol by a modification of the method of Okamoto *et al.* (*21*).

When the annealed DNA-RNA hybrids collected from the CsCl gradient were assayed without prior ribonuclease treatment (Fig. 2) the results were approximately similar to those shown in Fig. 1, as were the results of a similar experiment without either ribonuclease treatment or acid precipitation. A distinct peak of radioactivity was evident only when the H strand was used, and this fact supports the concept of possible contamination (Fig. 1).

To eliminate the objection that the L strand of SP8 DNA is intrinsically incapable of hybridizing with complementary RNA, each of the fractionated strands of SP8 DNA was annealed with RNA synthesized by highly purified RNA polymerase from *Escherichia coli* primed by both the L and the H strands of SP8 DNA. The results indicated that the RNA's are renatured predominantly with the purified DNA strand that served as their directive template. Not enough complementary RNA synthesized in vitro and characterized with respect to molecular weight was available to determine the proportion of homologous to heterologous DNA-RNA helix formation.

The extent of hybridization of the fractionated strands was determined with agar gels containing DNA (*10*). This technique was also used to detect host-specific mRNA in the RNA isolated from the phage-infected *Bacillus subtilis* (*12*). This method (*10*) minimizes the renaturation of DNA by trapping denatured DNA of high molecular weight in the agar, and it permits more efficient and preferential renaturation with complementary RNA. The results showed that only the H strand of the DNA from phage SP8 directs the formation of complementary RNA that can be isolated from bacteriophage-infected cells. We used this technique (*12*) also to show that mRNA complementary to host DNA is formed in SP8-infected *B. subtilis* and we obtained results consistent with the observation that infection with SP8 causes very little destruction of host DNA and does not interfere with the synthesis of total RNA measured chemically (*13*).

Clearly, phage DNA introduces into the host genetic information not already present; part of this information is contained in the phage-specific mRNA which is formed within minutes of infection. In the case of the well-described T-even phage system in *Escherichia coli,* early mRNA must act as a template for the synthesis of the numerous enzymes (*14*) which arise *de novo* in the bacterium and can account for the synthesis of unusual components in the bacteriophage DNA. Comparable enzymes have been found in *Bacillus subtilis* infected by bacteriophage PBS2 (*15*), and these can account for the substitution of thymine by uracil in this phage. A phage-induced enzyme, deoxycytidylate deaminase, has recently been observed in this laboratory (*16*) in extracts of *B. subtilis* infected with SP8. Activity is absent in uninfected cells but becomes detectable 8 minutes

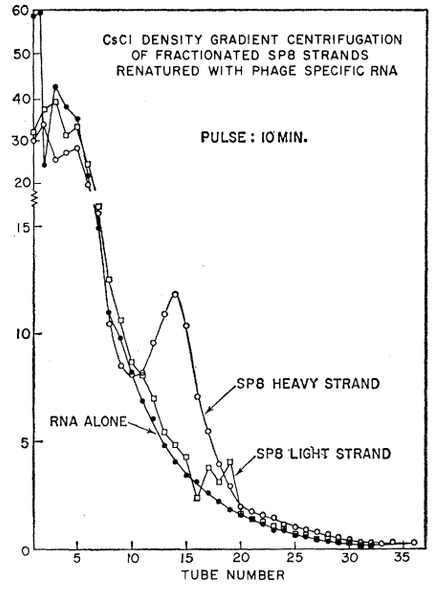

Fig. 2. Specificity of hybridization of RNA isolated from SP8-infected *B. subtilis* with the separated strands of SP8 DNA. Total acid-precipitable radioactivity. Ordinate: Radioactivity (count/min) of RNA labeled with H^3-uridine (after hybridization with fractionated phage DNA) of the CsCl density gradient fractions, precipitated with trichloroacetic acid. Abscissa: Fractions collected after preparative centrifugation. Hybridizations and centrifugations were performed by the procedures described in the legend to Fig. 1. The RNA labeled with H^3-uridine contained 90 count/min per microgram. The labeling time was from 4 to 14 minutes after infection. The L strand was further purified by a second passage through a column containing methylated bovine serum albumin. The total volume of hybridization mixtures was 0.76 ml. The drops collected from the centrifuge tubes were diluted to 2 ml with water and calf-thymus DNA, precipitated with trichloroacetic acid, and counted.

after infection and by the 15th minute reaches a maximum level comparable to that of the most active enzymes observed in *E. coli* infected with T-even phage. It seems likely that this phage-specific enzyme is responsible for supplying the deoxyuridylic acid that ultimately enters the 5-hydroxymethyluridylate in DNA from bacteriophage SP8. Thus it is apparent that host-specific mRNA and phage-specific mRNA can be synthesized in the same cell. Whether both or only one of the DNA strands of *B. subtilis* acts as a template for mRNA synthesis has not been determined in this study.

Other evidence, suggesting the transcription in vivo of only one of two DNA strands, comes from studies of other bacteriophage systems. Bautz and Hall (*17*) observed that the RNA formed after infection with T4 bacteriophage has a base composition such that adenine is not equal to uracil and cytosine is not equal to guanine. In an experiment similar to that reported here, Hayashi *et al.* (*18*) noted that the messenger RNA induced by infection with bacteriophage ΦX174 does not hybridize with the DNA from the mature bacteriophage particle but does hybridize with the double-stranded replicating form of ΦX174 DNA (*19*).

J. MARMUR
C. M. GREENSPAN
Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts

References and Notes

1. E. K. F. Bautz, *Proc. Natl. Acad. Sci. U.S.* **49**, 68 (1963); in *Informational Macromolecules*, H. J. Vogel *et al.*, Eds. (Academic Press, New York, 1963), pp. 409–416.
2. S. P. Champe and S. Benzer, *Proc. Natl. Acad. Sci. U.S.* **48**, 532 (1962).
3. E. P. Geiduschek, T. Nakomoto, S. B. Weiss, *ibid.* **47**, 1405 (1961); J. Hurwitz and J. T. August, *Progr. Nucleic Acid Res.* **1**, 59 (1963).
4. W. R. Romig and A. M. Brodetsky, *J. Bacteriol.* **82**, 135 (1961).
5. R. G. Kallen, M. Simon, J. Marmur, *J. Mol. Biol.* **5**, 248 (1962).
6. N. Sueoka and Ts'ai-Ying Cheng, *ibid.* **4**, 161 (1962).
7. F. Kahan and J. Hurwitz, unpublished results.
8. S. Cordes, H. T. Epstein, J. Marmur, *Nature* **191**, 1097 (1961); J. Marmur and S. Cordes, in *Informational Macromolecules*, H. J. Vogel *et al.*, Eds. (Academic Press, New York, 1963), pp. 79–87.
9. B. D. Hall and S. Spiegelman, *Proc. Natl. Acad. Sci. U.S.* **47**, 137 (1961); S. Spiegelman, in *Informational Macromolecules*, H. J. Vogel *et al.*, Eds. (Academic Press, New York, 1963), pp. 27–54.
10. E. T. Bolton and B. J. McCarthy, *Proc. Natl. Acad. Sci. U.S.* **48**, 1390 (1962); B. J. McCarthy and E. T. Bolton, *ibid.* **50**, 156 (1963).
11. D. Giacomoni and S. Spiegelman, *Science* **138**, 1328 (1962).
12. M. Mandel, unpublished results.
13. E. Palecek, unpublished results.
14. M. J. Bessman, *Molecular Genetics* (Academic Press, New York, 1963), pp. 1–64.
15. F. M. Kahan, *Federation Proc.* **22**, 406 (1963).
16. B. Riddle and F. M. Kahan, unpublished results.
17. E. K. F. Bautz and B. D. Hall, *Proc. Natl. Acad. Sci. U.S.* **48**, 400 (1962).
18. M. Hayashi, M. N. Hayashi, S. Spiegelman, *ibid.*, in press.
19. Aided by grants from the U.S. Public Health Service, National Aeronautics and Space Administration, and the National Science Foundation. Publication No. 228, graduate department of biochemistry. We thank Drs. F. M. Kahan and M. Mandel for many helpful discussions.
20. M. Nomura, K. Matsubara, K. Okamoto, R. Fujimura, *J. Mol. Biol.* **5**, 535 (1962).
21. K. Okamoto, Y. Sugino, M. Nomura, *ibid.*, p. 527.

3 July 1963

Malate Dehydrogenase: Multiple Forms in Separated Blastomeres of Sea Urchin Embryos

Abstract. *Sea urchin embryos at the 64-cell stage were dissociated by treatment with trypsin and separated by centrifugation on a sucrose gradient. The large blastomeres have two and the small blastomeres have three bands of* L-*malate dehydrogenase activity, which are separated by disk microelectrophoresis on polyacrylamide gel, whereas unfertilized eggs have five.*

Changes in the number and amounts of the multiple forms of enzymes in a given tissue, during the latter part of embryonic development, have been demonstrated for lactate dehydrogenases (*1*, *2*) and for malate dehydrogenases (*2*). The relationship of this phenomenon of multiple molecular forms of an enzyme to the "one gene-one enzyme" theory and to the problem of cellular differentiation is of interest. All the tissues of adult echinoderms that have been tested contain malate dehydrogenases which have characteristic ratios of activity with nicotinamide-adenine dinucleotide (NAD) and its analogs (*3*). Electrophoresis on either starch granules or gel, or adsorption to and elution from diethylaminoethanol (DEAE) cellulose columns, revealed three to five molecular forms of malate dehydrogenase in these tissues (*4*, *5*). Unfertilized eggs of the sea urchin, *Arbacia*, have five NAD-malate dehydrogenases, 6-hour embryos have three, and 12- to 48-hour embryos have four. We have now investigated the multiple forms of malate dehydrogenase in separated blastomeres of early embryos.

Eggs and sperm, collected by electric shocks (10 v), were washed and diluted for fertilization. Embryos were grown at 25°C to the 64-cell stage, collected by gentle centrifugation and frozen briefly. They were suspended in a small volume of 0.53*M* NaCl containing $2 \times 10^{-3}M$ ethylenediamine tetraacetate, *p*H 5.0, for 3 minutes at 37°C to remove the egg membranes and jelly. Two volumes of 0.265*M* Tris buffer, *p*H 7.9, containing 0.01 percent recrystallized trypsin were added and the mixture was placed in a glass homogenizer in a water bath maintained at 37°C. Gentle movements of the pestle were continued until microscopic examination revealed that the blastomeres were completely dissociated. The dissociated blastomeres were layered on a 0.29 to 0.87*M* sucrose gradient and centrifuged at 750*g* for 25 minutes. The large blastomeres formed a pellet at the bottom of the tube and the small blastomeres formed a layer between the 0.29 and 0.87*M* sucrose. The layers were separated and examined microscopically, then the cells were distintegrated by ultrasonic techniques to make the enzymes soluble.

The resulting preparations were assayed spectrophotometrically for the rate of reaction of malate dehydrogenase with NAD and its analog, by means of a Beckman DB spectrophotometer and a recording potentiometer (*5*). Aliquots of each preparation were subjected to disk microelectrophoresis on polyacrylamide gels for 70 minutes at 5 mA in tris-glycine buffer, *p*H 8.6. Positions of malate dehydrogenase activity were located by staining the gels in 10 ml of a solution containing: 0.05*M* L-malate; 0.001*M* NAD; 0.002*M* KCN; 0.0005*M* $MgCl_2$; 0.05*M* glycyl glycine; 0.05 mg/ml phenazine methosulfate; and 0.3 mg/ml nitro blue tetrazolium. The *p*H of the solution was 7.4.

Table 1. Malate dehydrogenase (MDH) activity in eggs and embryos of the sea urchin, *Arbacia punctulata*, as determined by spectrophotometry (column 2) and electrophoresis (column 3).

Stage of development	Ratio of activity with APAD/NAD	Bands of MDH activity	
		With NAD	With APAD
Unfertilized eggs	0.68	5	8
Whole embryos (6 hours)	0.63	3	
Whole embryos (48 hours)	2.2	4	
Small blastomeres	1.4 ± 0.13*	3	2
Large blastomeres	2.3 ± 0.20*	2	1

*The mean value of eight experiments ± standard error.