*THE MUTAGENIC ACTION OF NITROUS ACID ON "SINGLE-STRANDED" (DENATURED) HEMOPHILUS TRANSFORMING DNA**

By E. Edith Horn† and Roger M. Herriott

THE JOHNS HOPKINS UNIVERSITY SCHOOL OF HYGIENE AND PUBLIC HEALTH, BALTIMORE

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The mutagenic action of nitrous acid has been observed *in vivo*[1-3] and in a number of *in vitro* systems including tobacco mosaic virus,[4] bacteriophage,[5-10] poliomyelitis virus,[11] Newcastle's virus[12] and pneumococcal[13, 14] and subtilis[15, 16] transforming DNA. Earlier attempts in this laboratory and those recently reported by Stuy[17] to produce new markers in hemophilus DNA with nitrous acid were unsuccessful. In the face of positive results in two other systems, it was difficult to account for the failure in hemophilus, but a consideration of some of the possibilities led us to treat "single-stranded" (denatured) hemophilus DNA with nitrous acid and after renaturation[18, 19] to look for new markers. Such experiments are described below and show that in contrast to the failure with native DNA, nitrous acid-treated "single-strand" DNA when renatured converted large numbers of cells to a variety of antibiotic resistances. At the moment, it is not clear whether the deaminating action of nitrous acid produced new markers in the treated DNA or some chemical change made the DNA a mutagenic agent which on entering the cell produced a new marker in the host's genome by nongenetic processes.

Experimental.—Transformation procedures: The general methods of preparing and assaying *Hemophilus influenzae* transforming DNA have been described elsewhere.[20] In some instances, particularly in Figures 1 and 2, the DNA concentration in the transformation mixture was higher (0.5 and 1.0 μg/ml) than usual. In Table 2 it was 0.01 μg/ml. At 0.01 μg/ml and lower levels, the transformants are proportional to the DNA concentration. The high level (1.0 μg/ml) was used in the early experiments in order to detect small increases in numbers of new transformants.

Antibiotic resistances: Resistance profiles (surviving cells versus antibiotic concentration) were determined on wild type receptor cells for each antibiotic. These showed the typical precipitous drop in surviving cells and then "tailed" off below 10^{-5} survivors. Antibiotic levels which permitted cells with such marginal levels of resistance to survive were used to screen for induced markers.

In the case of streptomycin and kanamycin profiles of wild type cells, there was no flat region indicating considerable variation in resistance of the surviving cells. This was also the case among cells transformed with nitrous acid-treated DNA. However, the cells within a colony were uniform in their resistance even though the flat region was quite narrow.

The levels of antibiotics used and the approximate proportion of resistant cells in the sensitive untransformed cells are given in Table 1.

TABLE 1

Antibiotic	Streptomycin	Kanamycin	Viomycin	Erythromycin	Cathomycin (novobiocin)
Symbol	S	K	V	E	C
μg/ml on plates	5	8	150	4	0.5
Proportion of naturally resistant cells in the sensitive cells	4×10^{-6}	6×10^{-6}	3×10^{-6}	1×10^{-8}	1×10^{-7}

Denaturation of DNA: Denaturation of the DNA was obtained by heating 1 ml aliquots of purified DNA in 0.15 *M* NaCl-0.014 *M* Na_3 citrate for four minutes at 100°C, followed by rapid chilling in ice and water. The residual transforming activity for the marked DNA varied from

3 to 10 per cent of the initial value with the higher figure remaining when 100–200 μg/ml was denatured.

Treatment with nitrous acid: A solution of heated and chilled *Hemophilus influenzae* DNA was added to an equal volume of buffered sodium nitrite. The final concentrations of components were as follows: acetic acid, 0.035 *M;* Na acetate, 0.015 *M;* NaCl, 0.15 *M;* $NaNO_2$ and DNA as specified in individual experiments. The initial pH of this reaction mixture was 4.2–4.7, depending on the concentration of sodium nitrite (0.05 to 1.0 *M*). During incubation at 37°C, the pH rose as was experienced by Boeye,[11] probably as a result of decomposition of nitrous acid. At intervals, aliquots of the reaction mixture were withdrawn and neutralized to pH 7.2 with Na_2HPO_4. With the DNA concentration adjusted to 5 μg/ml, the solution was warmed to 66°C and held for an hour to renature the DNA, after which it was cooled gradually to room temperature over a half-hour period.

Controls: In general, background numbers of antibiotic-resistance cells in the receptor cell population were deducted from the experimental values. These background numbers did not change with the composition of the reaction medium nor in the acid controls in which the DNA was exposed to the acetate buffer in the absence of nitrite. The background numbers of resistant cells did not vary with the quantity of untreated wild type DNA, showing that the contribution from this source was negligible.

Recovery of the initial genetic markers by annealing[19] the denatured DNA varied from 30–60 per cent of the undenatured sample. Incubation of denatured DNA in the acetate buffer without nitrite, after which it was renatured, reduced the recovery of the initial marker a little more (a factor of 2 in 6 hr). The per cent residual transforming activity as shown in Figures 1 and 3 was calculated relative to such controls.

Results.—Effect of time and nitrite concentration: Figure 1 contains the results

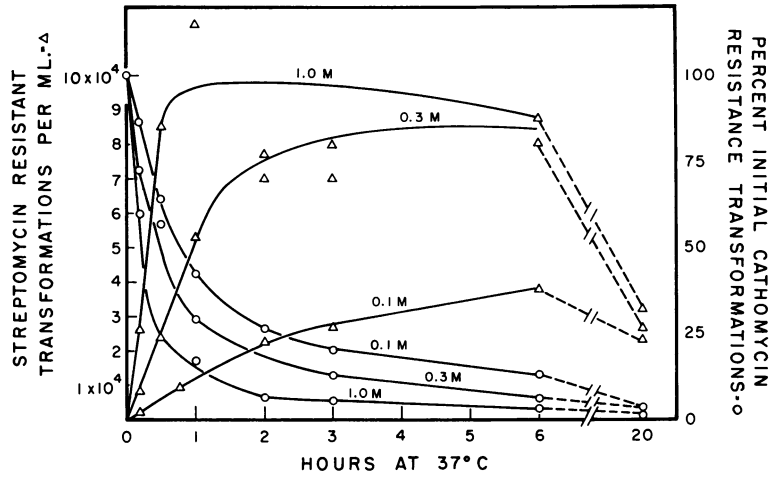

FIG. 1.—Effect of time and nitrite concentration on formation of streptomycin resistance transforming markers. The nitrite concentrations are indicated on the curves. The procedures are described in the *Experimental* section. DNA concentration in the transforming mixture was 1.0 μg/ml. The transformations noted are net increases since background values have been subtracted from all samples. Intrinsic cathomycin resistance marker is indicated by open circles.

of a representative experiment on the *formation* of streptomycin resistance markers in heat-denatured DNA by the action of nitrous acid. The number of cells becoming resistant to 5 μg/ml of streptomycin after being transformed with DNA that had been treated with nitrous acid and then renatured increased both with time of treatment (dose) and with nitrite concentration. The initial or intrinsic

marker (cathomycin resistance) of this DNA was gradually destroyed under these conditions. This is shown by the descending curves which use the scale on the right-hand ordinate of the chart. Generation of the streptomycin markers and destruction of the initial cathomycin marker increased as the nitrite concentration rose from 0.1 *M* to 1.0 *M*. Both changes were linear with time, in the initial stages, pointing to single-hit processes. Similar findings were reported earlier[4-8] for other systems in which mutations were produced by nitrous acid.

Effect of temperature: The rate of marker formation in "single-stranded" DNA increased as the temperature of the reaction mixture was raised. A Q_{10} of 2–3 was obtained for the reaction from initial rates at 25, 37, 55, 65, and 75°C. No experiments were made at higher temperatures.

Formation of different markers: Preliminary tests had shown that antibiotic resistances other than streptomycin resistance had been induced in the DNA. In order to permit a simple comparison of the formation of all such markers, denatured *unmarked* (wild type) DNA was exposed to nitrous acid. Aliquots were withdrawn from the molar nitrite mixture, the acid was neutralized, the sample was annealed, and, after appropriate dilution, aliquots were added to competent receptor cells for transformation. After the usual period for uptake and integration of the DNA, the cells were assayed for resistance to each of five different antibiotics. The results are shown in Figure 2. It may be seen that in DNA which carried *no*

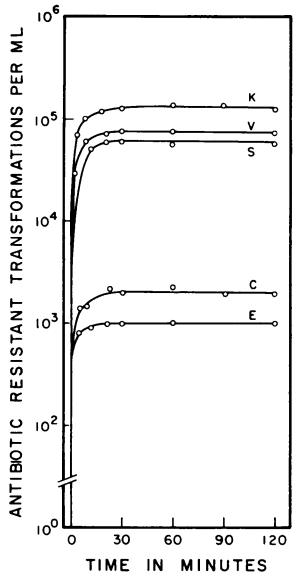

FIG. 2.—New antibiotic resistance markers produced by nitrous acid in unmarked DNA. $NaNO_2$ concentration = 1 molar. DNA concentration in transforming system was 0.5 μg/ml; K = kanamycin; V = viomycin; S = streptomycin; C = cathomycin (novobiocin); E = erythromycin.

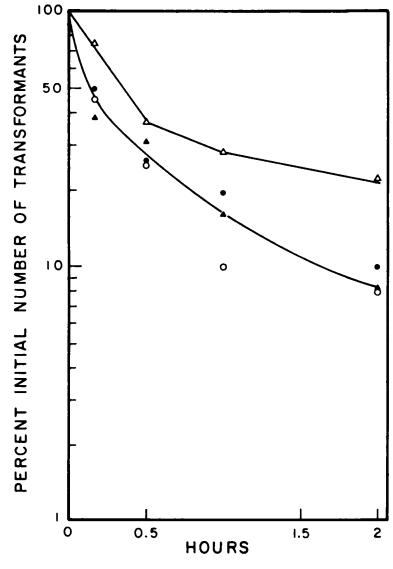

FIG. 3.—Sensitivity to nitrous acid (molar nitrite) of native progeny marker DNA derived from nitrous acid-generated markers. DNA in reaction mixture = 50 μg/ml. DNA in transformation system = 0.5 μg/ml. Open circles = isolate 1 from a streptomycin plate. Closed circles = isolate 2 from a streptomycin plate. Open triangles = isolate 1 from a kanamycin plate. Closed triangles = isolate 2 from a kanamycin plate.

genetic marker different from those in the receptor cells, nitrous acid produced large numbers of transformable genetic markers to all five different and independent antibiotics. In general, the new markers were independent, for a transformed cell was resistant to only one antibiotic but 10–15 per cent of the cells resistant to streptomycin or viomycin were also resistant to the other. No mutation to protoporphyrin utilization was detected.

Examining, in some detail, DNA treated for 30 min with molar nitrite led to the data shown in Table 2. In this experiment, the concentration of the treated DNA

TABLE 2

ANTIBIOTIC RESISTANCES PRODUCED BY HNO_2-DNA

HNO_2 Treatment: 50 μg/ml denatured DNA,† 1 *M* $NaNO_2$, *M*/20 acetate buffer pH 4.5, 37°C, 0.5 hr. Dilute 1:10 in phosphate buffer to bring to pH 7 ± 0.2; add NaCl to 0.3 *M*, incubate at 66°C for 1 hr and cool.
Trans. Mixture: 2 × 10^8/ml competent cells in brain heart infusion; 0.01 μg/ml DNA; shake 2 hr** at 37° C.
Plating: Trans. mixture diluted in broth and mixed on plate with antibiotic in agar plus growth medium.

				Resistant Colonies			
	Anti-biotic	Dilution for plating	Bacteria plated (total)	(1) Background	(2) After exposure to 0.01 μg/ml	(3) Trans./μg DNA*	(4) Relative increase (2)/(1)
HNO_2-DNA	S	1:100	2×10^7	109	303	2×10^6	2.8
				117	307		
	K	1:100	2×10^7	154	390	2.5×10^6	2.8
				150	396		
	V	1:100	2×10^7	133	323	2.4×10^6	2.8
				123	360		
	E	1:2	1×10^9	14	86	1.4×10^4	7.2
				7	74		
	C	1:2	1×10^9	46	176	1.9×10^4	2.9
				72	106, 235		
Controls‡	S	1:100	2×10^7	128	560	4.3×10^6	4.4
	K	1:100	2×10^7	154	826	6.7×10^6	5.4

* This figure represents the *net* increase in resistant colonies multiplied by the plating dilution and adjusted to 1 μg/ml DNA.
** Shaking 2 hr raised the cell population 10-fold including the background resistant cells in (1) but the cells made resistant by treated DNA increased less than 2-fold in this time, due perhaps to the slow integration of these markers. The effect of treated DNA is therefore greater than the data indicate.
† Wild type or cathomycin resistance DNA.
‡ Transformants with DNA from progeny of above S or K resistance HNO_2-DNA.

in the transformation mixture was reduced to 0.01 μg/ml, a level at which the transformants are proportional to the DNA concentration. The number of background resistant cells, being a component of the assay only and not a function of the chemical treatment, was subtracted from experimental figure, the difference appropriately multiplied by the dilution prior to plating and adjusted to one microgram of DNA in all instances. This figure in column 3 of Table 2 reached 2×10^6 or more for three of the five antibiotic resistances. This figure may be better appreciated by comparing it with the transforming capacity of DNA from cells carrying such markers. Tests of two such DNAs which had been put through the same denaturation, acid incubation, and renaturation treatment as the experimental but with sodium chloride replacing nitrite are denoted as controls in Table 2. These control DNAs produced only twice as many transformants as did the nitrous acid-treated DNA.

Litman[14] found a reduction in DNA uptake by competent cells as nitrous acid treatment progressed, which suggests that the above figure will be still higher when corrected. The customary correction for marker destruction by the mutagenic

agent has not been included for reasons that will be discussed later. *Even without these corrections,* the number of transformants produced by nitrous acid is astonishingly high, so high in fact that an alternative to the obvious interpretation is considered later in this paper. Litman and Ephrussi-Taylor[13, 14] had also found large numbers of new markers following nitrous acid treatment.

In comparing the effect of an agent on various mutable processes, Northrop[3, 36] has compared the number of mutant cells obtained after treatment to the number in the untreated controls. Such a comparison is shown in column 4 of Table 2. It is clear that despite considerable difference in the proportion of some markers, the ratio is remarkably constant. Only the "E" is different. Further discussion of this will be taken up later.

The resistance of nitrous acid generated markers to inactivation by this agent: The results in Figures 1 and 2 show that once the number of new markers reaches the maximum, continued exposure to nitrous acid produced surprisingly little inactivation. This plateau may represent an equilibrium between formation and destruction of markers but such an explanation is not easily reconciled with the variety of markers and the spread in conditions. This insensitivity to continued treatment was totally unexpected since denatured DNA has many more exposed amino groups to react than native DNA,[21] and similar results were obtained at elevated temperatures where intrastrand hydrogen bonding would be small. To establish that the resistance to nitrous acid was not illusory, the DNA in a reaction mixture was dialyzed free of nitrite and then re-exposed to fresh acid-nitrite. This produced nothing comparable to the rapid inactivation that is observed in natural markers (see Figs. 1 and 3). The difficulty of visualizing how extensive or continued deamination could fail to alter a number of genetic markers plus the problem of explaining the extraordinary number of so many new markers led us to consider an alternative possibility which will now be described.

Does nitrous acid make a mutagenic agent of DNA? It appears that both inactivation of the natural marker and formation of new markers are dependent on nitrous acid concentration, time, and temperature. It does not follow, however, that both effects are products of deamination. The new markers could have been produced by a side or minor reaction of nitrous acid on some structures of denatured DNA which made it a chemical mutagen. Besides providing the necessary chemical structure for such a mutagen, the DNA also provides the means of getting the mutagenic structure into the cell and carried to the host's genome where it can produce its effect—presumably at any of a number of loci. This would be more convincing if the treated DNA acted across specie barriers, e.g., nitrous acid-treated hemophilus DNA induced mutations in pneumococci, but negative results would not rule out such a proposal.

In support of the more expected mechanism of direct deamination of amino groups, it should be noted that the rate of destruction of natural markers decreases considerably with time of exposure (see Figs. 1 and 3). After the intrinsic genetic marker was reduced to about 10 per cent, this residual activity was destroyed more slowly and resembles in this respect the new markers formed by nitrous acid. This suggests that perhaps the insensitivity of nitrous acid-formed markers to the destructive action of this agent is not a peculiarity that compels one to seek an alternative explanation of this phenomenon.

Calculating the proportion of mutants: It is customary among workers in the field of genetics when calculating the proportion of mutants following treatment with agents which are also destructive to correct for the destructive action of the mutagen.[22, 23] This carries the assumption that the modified hereditary unit has the same sensitivity to the destructive action of the agent as the untreated and surviving units. As noted in the previous sections of this paper, this is not observed in the present study. The treated DNA is insensitive to further action of nitrous acid whereas naturally occurring markers and progeny markers of those formed from nitrous acid are sensitive to destruction by this agent (see Fig. 3). If it is assumed that new markers are being produced with nitrous acid and not a mutagenic agent, then several conclusions emerge from these results. The modified (deaminated) DNA is importantly different not only from the DNA from which it was derived, but also from its progeny. The assumption regarding the sensitivity of the modified DNA to the mutagenic agent which is commonly made when calculating a corrected proportion of mutants is *not* valid in the present instance. Finally, the present results support a conclusion which became apparent from the work with genetically potent bromouracil DNA,[24, 25], namely, that nucleic acids with different chemical properties can produce progeny that appear identical. In the present instance, new markers to antibiotic resistance which were insensitive to nitrous acid, produced markers which were sensitive to this agent.

Discussion.—The contrast in results of the action of nitrous acid on native and denatured hemophilus DNA and again between native hemophilus[17] and pneumococcal[13, 14] and subtilis[15, 16] DNAs is so striking that an understanding of the basis of these differences was sought through a series of experiments. However, as yet no satisfactory explanation covers all cases. Cross linkage of strands by nitrous acid[17, 26] would account for the failure of native hemophilus DNA to yield new markers if its replication requires strand separation,[27] but then such linkage would have to be specie-specific, for no such problem in detecting new markers exists in similarly treated native pneumococcal DNA[13] or in *E. coli* phages T2 and T4.[5–10] Heating nitrous acid-treated native hemophilus DNA to 100°C which might break weak cross-linking bonds and allow strand separation did not raise its intrinsic transforming activity after renaturation.

If the pneumococcal or subtilis DNAs were prepared from rapidly growing cells, they might have contained some "single-stranded" DNA which accounted for formation of the new markers. Hemophilus DNA is usually prepared from cells in the stationary phase;[20] hence, it might have little or no "single-stranded" DNA. However, no new genetic markers were found when DNA isolated from rapidly multiplying hemophilus was treated with nitrous acid and tested with and without annealing.

If in pneumococcal DNA a few sugar phosphate bonds were opened in the DNA at widely separated points[28, 29] by the cellular DNAase at time of lysis,[30] they might have allowed the bases at these breaks to become deaminated, thereby producing new markers. However, mild DNAase treatment of native hemophilus DNA yielded no new markers with nitrous acid treatment.

The loosening of the hydrogen bonds in pneumococcal DNA at pH 4–5, where the nitrous acid was found to be effective, may be more pronounced than in hemophilus DNA but it would not appear to be due to a lower guanine-cytosine content.[31]

Lowering the pH of the reaction mixture to pH 4.0 was insufficient to produce new markers in native hemophilus DNA. Other explanations will be sought and tested, but they are not obvious at this time.

Schuster[21] has observed that denaturation of thymus DNA increases the rate of deamination of the six position amino groups of adenine and cytosine but not for the two position amino group of guanine. Schuster interprets this as meaning the two amino group of guanine is free in the native DNA. This information may be pertinent in suggesting the amino groups responsible for new markers, but it will be difficult to establish, for even the best transforming DNA is heterogeneous. Not more than about two per cent of the molecules in the purest DNA preparations from hemophilus carry any one particular marker. The remainder carry other markers.

The formation of large numbers of five different antibiotic markers is in sharp contrast to our failure to find new markers for protoporphyrin utilization, despite the latter's reasonably high (10^{-5}) natural proportion of mutants.[32] Clarification will have to await the screening for other markers to determine whether the high rate of appearance of new markers is the usual or unusual occurrence. Nonrandomness in mutagenesis is well established;[33] yet there is a large literature indicating that a general increase in mutations is the more common observation. It is clear from Column 4 of Table 2 that the ratio of new resistance markers to background numbers is remarkably constant for four of the five resistances and the fifth differed only by a factor of 2–3. Thus, within the group of independent antibiotic resistances the effect was relatively constant despite the variation of 30–100 fold in absolute numbers.

Cabrera's studies[34] in this laboratory with ultraviolet light on single-stranded DNA do not support the notion that antibiotic resistance markers are readily produced by any mutagenic agent. The presence of a number of antibiotic resistance sites, of which three are known for streptomycin,[35] favors their appearance but this is probably not the major explanation. The failure to produce protoporphyrin utilizing markers may mean simply that a different change in the code is needed from that in which an amino group is replaced by a hydroxy (or keto) group.

Summary.—(1) New antibiotic resistance markers were not observed following nitrous acid treatment of native hemophilus DNA. This confirms the recent report of Stuy and is in contrast with the positive results of other transforming DNAs. (2) Similar treatment of denaturated hemophilus DNA followed by renaturation yielded large numbers of transformable resistance markers to five different antibiotics. (3) The resistance of the newly formed markers to continued exposure to nitrous acid is difficult to explain. A small fraction of natural markers is also less sensitive to this agent, so there is a precedent for such insensitivity. The possibility has been considered that nitrous acid makes a mutagenic agent of DNA and that the new markers are generated by this mutagenic DNA acting on the genome of the recipient cell by a nongenetic chemical interaction. (4) The common practice of correcting the mutation rate on the assumption that the new markers have the same sensitivity as the natural markers was found to be invalid for this system. (5) Experiments failed to account for the difference in response of native pneumococcal and hemophilus DNAs to nitrous acid.

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[1] Kaudewitz, F., *Nature*, **183**, 1829 (1959).

[2] Zamenhof, S., *J. Bacteriol.*, **81**, 111 (1961).

[3] Northrop, J. H., and F. Cavallero, these PROCEEDINGS, **47**, 1602 (1961).

[4] Mundry, K. W., and A. Gierer, *Z. Vererbungslehre*, **89**, 614 (1958).

[5] Tessman, I., *Virology*, **9**, 375 (1959).

[6] Freese, E., in *Structure and Function of Genetic Elements*, Brookhaven Symposia in Biology, No. 12 (1959), p. 63.

[7] Vielmetter, W., and C. M. Wieder, *Z. Naturforsch.*, **14b**, 312 (1959).

[8] Vielmetter, W., and H. Schuster, *Biochem. Biophys. Res. Comm.*, **2**, 324 (1960).

[9] Bautz-Freese, E., and E. Freese, *Virology*, **13**, 19 (1961).

[10] Baylor, M. B., and H. R. Mahler, *Virology*, **16**, 444 (1962).

[11] Boeye, A., *Virology*, **9**, 691 (1959).

[12] Granoff, A., *Virology*, **13**, 402 (1961).

[13] Litman, R., and H. Ephrussi-Taylor, *C.R. Acad. Sci. (Paris)*, **249**, 838 (1959).

[14] Litman, R., *J. Chimie Physique*, **58**, 997 (1961).

[15] Nester, E., and J. Lederberg, these PROCEEDINGS, **47**, 52 (1961).

[16] Anagnostopoulos, C., and I. P. Crawford, these PROCEEDINGS, **47**, 378 (1961).

[17] Stuy, J., *Biochem. Biophys. Res. Comm.*, **6**, 329 (1961).

[18] Marmur, J., and D. Lane, these PROCEEDINGS, **46**, 453 (1960).

[19] Herriott, R. M., these PROCEEDINGS, **47**, 146 (1961).

[20] Goodgal, S. H., and R. M. Herriott, *J. Gen. Physiol.*, **44**, 1201 (1961).

[21] Schuster, H., *Biochem. Biophys. Res. Comm.*, **2**, 320 (1960).

[22] Demerec, M., and J. Hanson, in *Genes and Mutations*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 16 (1951), p. 215.

[23] Jensen, K. A., I. Kirk, G. Kølmark, and M. Westergaard, *ibid.*, p. 245.

[24] Zamenhof, S., R. De Giovanni, and K. Rich, *J. Bacteriol.*, **71**, 60 (1956).

[25] Szybalski, W., Z. Opara-Kubinska, Z. Lorkiewicz, E. Ephrati-Elizur, and S. Zamenhof, *Nature*, **188**, 743 (1960).

[26] Geiduschek, E. P., these PROCEEDINGS, **47**, 950 (1961).

[27] Watson, J. D., and F. C. D. Crick, *Nature*, **111**, 964 (1953).

[28] Dekker, C. A., and H. Schachmann, these PROCEEDINGS, **40**, 894 (1954).

[29] Thomas, C. A., Jr., *J. Am. Chem. Soc.*, **78**, 1861 (1956).

[30] McCarty, M., and O. T. Avery, *J. Exptl. Med.*, **83**, 97 (1946).

[31] Belozersky, A. N., and A. S. Spirin, in *The Nucleic Acids*, ed. E. Chargaff and J. N. Davidson (New York: Academic Press, 1960), p. 147.

[32] Hsu, Y. C., unpublished results in this laboratory.

[33] Benzer, S., in *The Chemical Basis of Heredity*, ed. W. D. McElroy and H. B. Glass (Baltimore: Johns Hopkins Press, 1957), p. 70.

[34] Cabrera, E., unpublished results.

[35] Hsu, Y. C., and R. M. Herriott, *J. Gen. Physiol.*, **45**, 197 (1961).

[36] Northrop, J. H., *J. Gen. Physiol.*, **43**, 541 (1960).