[16] Furth, J. J., J. Hurwitz, and M. Goldmann, *Biochem. Biophys. Res. Comm.*, **4**, 431 (1961).
[17] Stevens, A., in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 100.
[18] Reich, E., A. J. Shatkin, and E. L. Tatum, *Biochim. Biophys. Acta*, **53**, 132 (1961).
[19] Cohen, S. S., and H. Barner, these PROCEEDINGS, **40**, 885 (1954).
[20] Hurwitz, J., J. J. Furth, M. Anders, P. O. Ortiz, and J. T. August, in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 91.
[21] Furth, J. J., J. Hurwitz, R. Krug, and M. Alexander, *J. Biol. Chem.*, **236**, 3317 (1961).
[22] Preiss, K., M. Dieckmann, and P. Berg, *J. Biol. Chem.*, **236**, 1748 (1961).
[23] Wood, W. B., and P. Berg, these PROCEEDINGS, **48**, 94 (1962).
[24] Ning, C., A. Stevens, J. C. Loper, *Biochem. Biophys. Res. Comm.* **7**, 30 (1962).
[25] Tissières, A., D. Schlessinger, and F. Gros, these PROCEEDINGS, **46**, 1450 (1960).
[26] After this paper had been written, Goldberg and Rabinowitz[30] reported that actinomycin D inhibits DNA-linked RNA synthesis in HeLa cells.
[27] Maaløe, O., and N. Symonds, *J. Bact.*, **65**, 177 (1953).
[28] Jacob, F., and J. Monod, *J. Mol. Biol.*, **3**, 318 (1961).
[29] Gros, F., W. Gilbert, H. H. Hiatt, G. Attardi, P. F. Spahr, and J. D. Watson, in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 111.
[30] Goldberg, I. H., and M. Rabinowitz, *Science*, **136**, 315 (1962).

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*REACTIVATION AND HYBRIDIZATION OF REDUCED ALKALINE PHOSPHATASE**

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There is an abundance of evidence that the structural gene for a protein controls its primary amino acid sequence,[1, 2] and there is strong evidence that the folding of the polypeptide chain of ribonuclease is largely determined by the primary structure.[3] However, for relatively large proteins, especially those composed of more than one polypeptide chain, it is not clear whether folding and polymerization are entirely determined by primary structure or require additional information available within the cell. It has been suggested, for example, that both α chains of hemoglobin are made on the same ribosome, are released as a dimer, and combine in the cytoplasm with a similarly made β-chain dimer to form the complete hemoglobin molecule.[4]

As part of a study of the synthesis and polymerization of the separate peptides of a protein molecule, experiments have been carried out on the dimerization and concurrent restoration of enzymatic activity of alkaline phosphatase inactivated by reduction of disulfide bridges. This enzyme,[5] purified from *Escherichia coli*, has a molecular weight of 80,000 and in its native state appears to be a tightly folded globular molecule. It is resistant to proteolysis by trypsin and chymotrypsin and is stable at 85°C for at least 30 min in the presence of 10^{-2} M Mg^{++}. Enzymatic activity persists even after incubation in 6 M urea, but a combined treatment with urea and thioglycollic acid leads to reduction of disulfide bonds and makes the resultant sulfhydryl groups on the molecule accessible to alkylation by iodoacetic

acid. Alkylation leads to irreversible inactivation (denaturation). The denatured material is stable in solution as a structure of molecular weight 40,000.[6] Analysis of the composition of the peptides produced by tryptic digestion of the protein has demonstrated that the enzyme is composed of two subunits with apparently identical amino acid composition.[6] The native enzyme contains two zinc atoms per molecule[7] and binds approximately two phosphate ions.[8] Inhibition studies with chelating agents and phosphate strongly indicate that both the zinc and the phosphate binding sites are at or near the active site. This suggests that the dimer contains two active sites, but it is not known whether these sites are located on the individual monomers as such or form a bridge between them.

It was found that enzyme which had been reduced with thioglycollic acid in urea but not reacted with iodoacetic acid could be reactivated under oxidizing conditions. In this paper, we report the kinetics of the reactivation process, the production of hybrids between electrophoretically different mutant *E. coli* phosphatases, and the production of intergeneric hybrids between the phosphatase produced by *E. coli* and that produced by *Serratia marcescens*.

Methods and Materials.—Cell growth and enzyme purification: Cells were cultivated in a medium containing 0.12 *M* Tris, 0.08 *M* NaCl, 0.02 *M* KCl, 0.02 *M* NH_4Cl, 0.003 *M* Na_2SO_4, 0.001 *M* $MgCl_2$, 2×10^{-4} *M* $CaCl_2$, 2×10^{-6} *M* $ZnCl_2$, 0.5% glucose, and 0.5% Difco Bacto-peptone adjusted to pH 7.5. The peptone contained the only source of phosphate, in an amount such that growth of the culture became phosphate limited at a cell density of about 5×10^8 ml. After 5 hr of growth beyond the point of phosphate exhaustion, full enzyme production was achieved and the cells were harvested and washed in Tris buffer *M*/15 pH 9.0. A cell suspension of about 10^{10} cells/ml was held at 0°; sucrose was added to 0.5 *M*, lysozyme to 0.1 mg/ml, and ethylenedinitrilotetraacetic acid to 0.002 *M*. After 10 min in the cold, magnesium sulfate was added to 0.01 *M*, and the suspension was centrifuged at $12{,}000 \times g$ for 20 min. The supernatant of this partial protoplasting, which contained most of the alkaline phosphatase in the cells,[9] was dialyzed against 10^{-2} *M* Tris, pH 7.4, plus 10^{-3} *M* $MgSO_4$ and was then purified by gradient elution from DEAE cellulose. The material in the enzyme peak was at least 95% pure as judged by its specific enzymatic activity and by its behavior on starch gel electrophoresis.

Reduction: The protein was suspended in 6 *M* urea at a concentration between 2 and 10 mg/ml. After N_2 had been bubbled through the solution for 20 min, neutralized redistilled thioglycollic acid was added to a concentration of 0.175% and N_2 bubbling was continued for 4 hr. This material was frozen and stored at −20°C. Storage over a period of several months did not change the level of reactivation obtained.

Electrophoresis: Starch gel electrophoresis was carried out vertically by a modification of the method of Smithies[10] for four to six hr with a gradient of approximately seven volts per centimeter. The buffer used was 4×10^{-3} *M* Tris at pH 8.0. After electrophoresis the gels were sliced longitudinally. One half was stained for phosphatase activity by a modification of the Gomori histological stain[11] for alkaline phosphatase. The staining solution contained 75 mg Nuclear Fast Red B salt (National Aniline) and 25 mg α-naphthyl phosphate (Sigma) in a solution of 0.06% borax. After development of color, the gels were fixed in a solution containing five parts methanol, five parts water, and one part glacial acetic acid by volume. The second half of each gel was stained for total protein with a 1% solution of Buffalo Black NBR (National Analine) in the above fixative.

Enzymatic activity was measured by the rate of hydrolysis of *p*-nitrophenyl phosphate (NPP: Sigma), 0.02% in 1 *M* Tris pH 8.0, as previously described.[6]

Standard buffer for reactivation contained 10^{-2} *M* $Mg(C_2H_3O_2)_2$, 6×10^{-2} *M* KCl, 2×10^{-6} *M* $ZnCl_2$, and 6×10^{-3} *M* mercaptoethanol in 10^{-2} *M* Tris pH 7.8.

Pronase-P (*Streptomyces griseus* protease) B grade, was obtained from the California Corporation for Biochemical Research. The usual incubation period was 30 min at 45°C at a pronase concentration of 10 μg/ml in standard buffer.

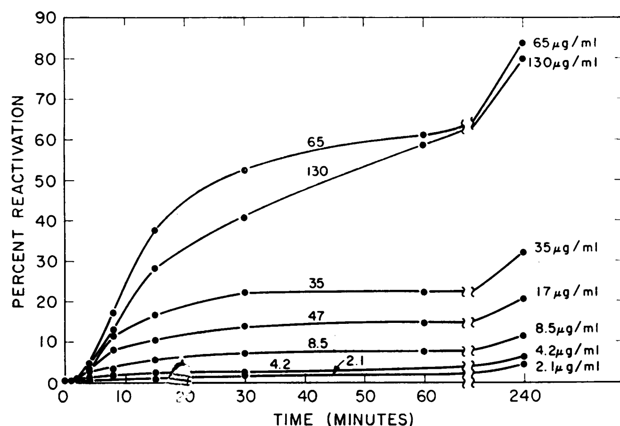

FIG. 1.—Recovery of enzymatic activity from reduced alkaline phosphatase as a function of time and protein concentration. Details in text.

Results.—The reactivation process: Purified alkaline phosphatase, which had been reduced as described under *Methods and Materials*, was diluted into standard buffer at 37°C, and the increase in enzymatic activity was measured as a function of time. Figure 1 illustrates reactivation at various protein concentrations, with enzymatic activity expressed as the per cent of activity which would be present if all the protein in the incubation mixture were native enzyme. After a lag of several minutes, enzymatic activity increases, reaching a maximum in approximately thirty minutes. The maximal rate of reactivation is obtained at a concentration of 65 µg/ml. At lower concentrations, both the rate of reactivation and the final level of activity are correspondingly reduced, indicating that a second irreversible competing reaction is taking place simultaneously. If we imagine the reactivation process as being the association of two inactive monomers to form an active dimer, mediated by disulfide bond formation, this second reaction might be the formation of those incorrect disulfide bridges which would lead to irreversible denaturation of the protein. Increasing the concentration to 130 µg/ml neither increases the rate nor abolishes the lag, indicating that both the lag and the rate of reaction are controlled, not by collision of monomers, but rather by the rate of folding and formation of correct secondary structure. This is further substantiated by the strong temperature dependence of the reactivation process shown in Figure 2. Although the collision frequency would be only weakly dependent on the temperature, the reaction does not proceed at a detectable rate at 0°C, even after pre-incubation at 37°C (Fig. 3). Pre-incubation at 0° does not affect the rate at which reactivation proceeds when the sample is warmed to 37°C.

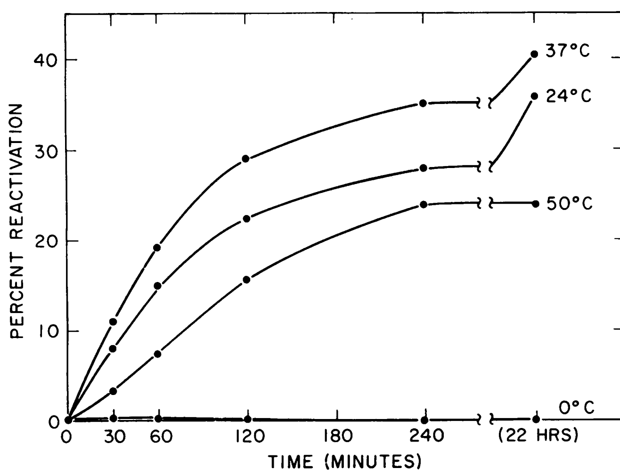

FIG. 2.—Recovery of enzymatic activity from reduced alkaline phosphatase as a function of temperature. Protein concentration is 65 µg/ml.

When mercaptoethanol is omitted from the standard

buffer, the amount of reactivation observed is reduced to a level which varies from zero to 50% of that observed in the complete standard buffer.

The maximum reactivation obtained in the standard buffer was about 80% of the original activity. The maximum was about 60% when the reactivating medium contained, in addition to the buffer, a crude extract of cell proteins at a concentration of 4 mg/ml. Thus the monomers are able to dimerize even in the presence of a high concentration of heterologous protein.

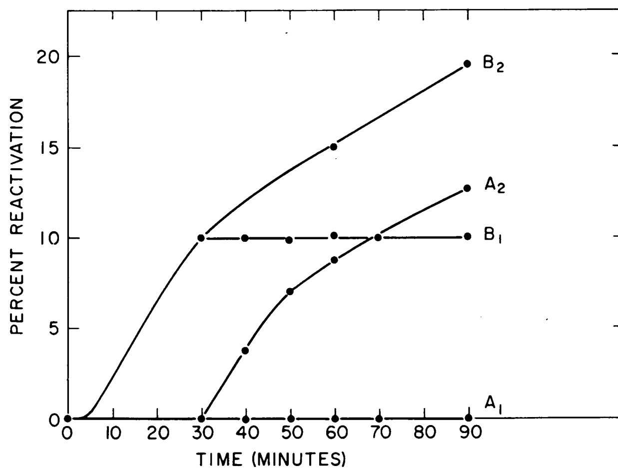

FIG. 3.—Recovery of enzymatic activity from reduced alkaline phosphatase. Protein concentration is 65 μg/ml. A_1 incubated at 0°C. A_2 incubated at 0°C. for 30 min, then at 37°C. B_1 incubated at 37°C for 30 min, then at 0°C. B_2 incubated at 37°C.

Properties of reactivated material: Both native enzyme and reactivated protein are stable for at least 30 min at 85°C in the presence of 10^{-2} *M* Mg^{++} and are resistant to proteolysis by pronase, which attacks the monomers and most of the other cell proteins at a very rapid rate. They behave identically when reacted with antiserum to native enzyme on Ouchterlony double diffusion plates and show the same pattern of elution from DEAE cellulose. Thus, the reactivated material appears to be the same as the native alkaline phosphatase by the above criteria.

Electrophoresis: Native alkaline phosphatase behaves as several electrophoretically different molecular species when the material is subjected to starch gel electrophoresis.[12] The different bands have approximately equal enzymatic activity per unit of protein. The phenomenon of multiple molecular electrophoretic forms of a single enzyme has been observed in several other systems.[13] Single mutations in the alkaline phosphatase structural gene which change the charge or the enzymatic activity of the protein affect all the bands identically, indicating that they are all the product of the same gene.[12] Although the nature of the banding phenomenon is not yet entirely understood, the factors responsible are unaffected by reduction and reactivation, since reactivated material exhibited the same banding pattern as native enzyme. The invariance of pattern was also true in the case of a preparation which had been enriched in a single band by fractionation on DEAE cellulose (Fig. 4*B*). This constitutes additional proof of the identity of the native and reactivated proteins.

Hybridization: From negative point mutations in the phosphatase structural gene, pseudo-revertants have been selected[2] which have full enzymatic activity, but produce enzyme protein which is electrophoretically different from normal enzyme. In some of these cases, the restoration of activity has been shown to be due to a second mutation within the structural gene which results in the formation of an altered but active protein.

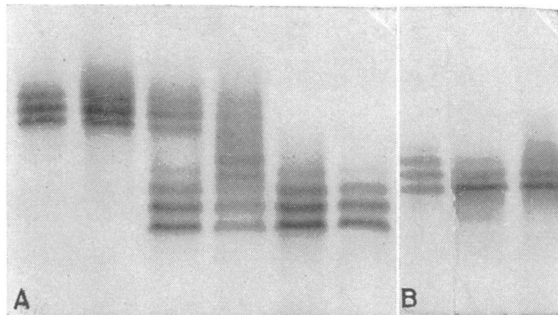

FIG. 4.—Starch gel electrophoresis. *A*. Hybridization of pseudo-revertants (from left): 1. Pseudo-revertant $U_{24}R_3A$, native. 2. Pseudo-revertant $U_{24}R_3A$, reduced and reactivated. 3. Pseudo-revertants $U_{24}R_3A$ and $U_{11}R_3A$; reduced and reactivated separately, then mixed. 4. Pseudo-revertants $U_{24}R_3A$ and $U_{11}R_3A$; reduced separately, mixed, then reactivated together. 5. Pseudo-revertant $U_{11}R_3A$, reduced and reactivated. 6. Pseudo-revertant $U_{11}R_3A$, native.

B. Invariance of pattern after reduction and reactivation (from left). 1. Native *E. coli* alkaline phosphatase. 2. Native *E. coli* alkaline phosphatase, enriched for first strong band. 3. Sample number 2, reduced and reactivated.

The alkaline phosphatases purified from two such pseudo-revertants, one which moves faster than the wild-type enzyme upon starch gel electrophoresis and one slower, were reduced and reactivated under the conditions described above. When the reactivated enzymes were mixed, no new bands appeared upon electrophoresis. However, when the reduced proteins from the two pseudo-revertants were combined and the reactivation was allowed to proceed in the mixture, bands of intermediate mobility appeared in addition to the bands characteristic of the pseudo-revertant enzymes (Fig. 4*A*), indicating the presence of a hybrid molecular species. The hybrid region showed three strong bands, as did each of the two native enzymes in the preparations used. The production of the hybrid species confirms the conclusion that reduction and reactivation of the enzyme do involve the separation of component monomers and the reformation of an active dimer.

Intergeneric hybridization: Experiments were performed using alkaline phosphatase from *Serratia marcescens* together with that from *E. coli*. The two organisms are classified in separate genera, and have respectively 58 and 50 mole per cent guanine plus cytosine in their DNA.[14] The enzyme produced by *S. marcescens* is similar in many respects to *E. coli* alkaline phosphatase.[15] The two enzymes are equally inhibited by inorganic phosphate; they are both stable at high temperatures and have approximately the same turnover number for hydrolysis of *p*-nitrophenyl phosphate. However, the amino acid sequence of these enzymes is very different, as demonstrated (1) by the difference in their isoelectric points, (2) by the fact that antiserum prepared against *E. coli* enzyme reacts with *S. marcescens* enzyme with less than 1 per cent of the affinity with which it reacts with the *E. coli* enzyme, and (3) by the fact that the fingerprints of the two enzymes after tryptic digestion show that most of their peptides are different. When the *E. coli* gene is introduced into *S. marcescens* by means of episomal transfer, it produces the normal *E. coli* enzyme, which, as previously reported, shows that the genetic code is read in the same way in these two organisms in spite of the different base composition of their DNA.[15]

When both the *S. marcescens* gene and the *E. coli* gene are present in active form (P^+) in the same cell, both enzymes are produced. In addition, enzymatically active bands are found with electrophoretic mobility intermediate between those of the two parental strains (Fig. 5*A*, number 5) at a position where a mixture of the two parental enzymes (Fig. 5*A*, number 3) shows no activity. This indicates that

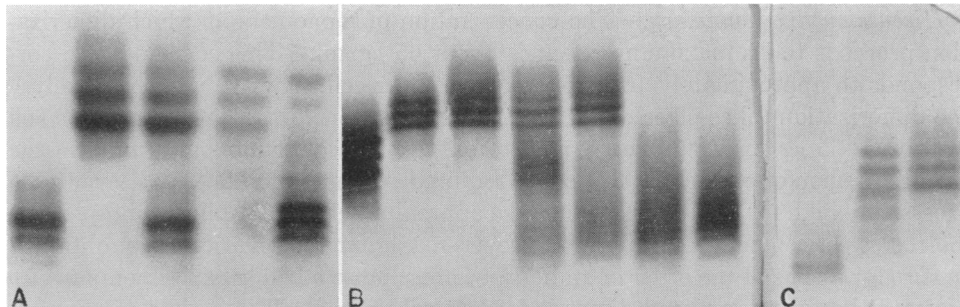

FIG. 5.—Starch gel electrophoresis. *A*. Patterns of *E. coli-S. marcescens* hybrid strains: 1. *S. marcescens,* wild type. 2. *E. coli,* wild type. 3. Mixture of numbers 1 and 2. 4. Hybrid: *S. marcescens* P^- with episome carrying *E. coli* P^+. 5. Hybrid: *S. marcescens* P^+ with episome carrying *E. coli* P^+.

B. Intergeneric hybridization (from left): 1. *E. coli* wild type, native. 2. *E. coli* pseudo-revertant $U_{24}R_3A$, native. 3. *E. coli* pseudo-revertant $U_{24}R_3A$, reduced and reactivated. 4. *E. coli* pseudo-revertant $U_{24}R_3A$ and *S. marcescens* wild type; reduced separately, then mixed and reactivated. 5. *E. coli* pseudo-revertant $U_{24}R_3A$ and *S. marcescens* wild type; reduced and reactivated separately, then mixed. 6. *S. marcescens* wild type, reduced and reactivated. 7. *S. marcescens* wild type, native.

C. Intergeneric complementation (from left): 1. *S. marcescens* wild type. 2. Hybrid, *S. marcescens* P^- with episome carrying *E. coli* P^+. 3. *E. coli* wild type.

within the cell a hybrid enzyme can be produced by the combination of one monomer of the *E. coli* type with one monomer of the *S. marcescens* type. The *S. marcescens* enzyme can be reduced and reactivated under the same conditions used for the *E. coli* enzyme, and it shows the same invariance of pattern upon electrophoresis after reactivation. We performed an *in vitro* hybridization experiment similar to that described in the preceding section (Fig. 4*A*), using *S. marcescens* phosphatase and enzyme from the fast moving pseudo-revertant. When the two enzymes were reduced separately and mixed and reactivation was allowed to proceed, enzymatically active bands with intermediate mobility were obtained (Fig. 5*B*), as was the case with the two *E. coli* pseudo-revertants. These results indicate that, either *in vivo* or *in vitro*, a dimer can be produced which contains one chain of the *E. coli* type and one chain of the *S. marcescens* type. Furthermore, they imply that, at least in the case of alkaline phosphatase, dimerization takes place after the monomers have left the ribosomes upon which they were synthesized.

Complementation: We have also used episomal transfer to introduce the *E. coli* phosphatase gene into an *S. marcescens* mutant (isolated by E. Lin using ethylmethane sulfonate as a mutagen) in which the level of *S. marcescens* phosphatase activity is less than one one-hundredth that of the wild-type (P^-). When an extract from the partial zygote is examined by electrophoresis, we see bands with mobility characteristic of *E. coli* enzyme. In addition, however, there are bands which move more slowly than those of the *E. coli* enzyme. These bands are in the precise position expected for an *E. coli- S. marcescens* hybrid, i.e., their mobilities correspond to those of the hybrid produced when both the *E. coli* and *S. marcescens* genes are functioning normally in the same cell and are *enzymatically active* (Fig. 5*A*, number 4, and Fig. 5*C*). Thus, the *E. coli* gene product has rescued the product of the *S. marcescens* gene to form active enzyme. This phenomenon is analogous to the genetic complementation observed in many other systems in which two different genes contribute to the formation of an active enzyme.

Discussion and Summary.—The concentration of monomers at which dimerization proceeds to its maximum extent is about 65 μg/ml. This concentration corresponds to approximately 100 monomer units per cell. But if, as seems likely,[16] the dimerization takes place either at the cell membrane or between it and the cell wall, then 65 μg/ml would correspond to only ten or fewer monomer units in the essential region of each cell. Thus, the required concentration does not seem to be excessively high for a cell which can make approximately half a million phosphatase molecules per cell per generation. However, maximum reactivation is not obtained *in vitro* for a time of the order of an hour, whereas pulse labeling experiments *in vivo* indicate that the completed dimer is formed within the cell in a time of approximately five seconds.[16] Thus, the *in vitro* conditions for dimerization differ considerably from those within the cell. The higher *in vivo* rate probably does not reflect a higher collision frequency, because higher concentrations *in vitro* do not result in more rapid reactivation. In addition, calculations of the collision frequency indicate that two molecules of the size of the phosphatase monomers would collide randomly with a frequency which is many orders of magnitude greater than the rate observed in the reactivation process.

Since four sulfhydryl residues can be titrated with iodoacetic acid on each reduced monomer,[17] it is likely that reactivation requires the reformation of at least some disulfide bonds. There is no evidence to indicate whether any of these disulfide bonds are between chains, or whether they are all within the same chain and are necessary for a specific monomer configuration which allows the formation of a stable molecule. The fact that the reactivation process does not occur in the absence of mercaptoethanol suggests that disulfide bonds leading to inactive configurations can form *in vitro* and that, with the help of mercaptoethanol, disulfide exchanges can occur with sufficient frequency to allow the enzyme to come to its most stable configuration. The same conclusions were reached by Anfinsen *et al.*[3] from their experiments on the reactivation of reduced ribonuclease.

Very little information is obtained from these experiments as to the nature of the multiple banding found in starch gel electrophoresis. Evidently, the peptide chain which is controlled by the structural gene can exist in several alternate states with respect to net charge, perhaps due to the addition of one or more small molecules without appreciable variation in molecular weight. However, the nature and function of these alternate states is not yet understood.

Both *in vivo* and *in vitro*, enzymatically active hybrids are found, the subunits of which are determined by different genes and therefore synthesized on different templates. This result supports the hypothesis that genetic complementation can result from the action of two different structural genes, each producing one monomer of an enzymatically active hybrid, as suggested by Fincham[18] and Brenner.[19]

The formation of an active *E. coli-S. marcescens* hybrid indicates that these proteins possess similar secondary structure, despite their compositional and immunological differences. In view of the different nucleotide composition of the DNA of the two organisms, this leads to the hypothesis that the only mutations in the alkaline phosphatase gene which survived the evolutionary changes in DNA nucleotide sequence were those in which enzymatic activity, and therefore the secondary structure of the enzyme, remained substantially unaltered. This is not surprising in view of the ease with which one obtains *E. coli* pseudo-revertants produc-

ing altered but fully active enzymes which are genetically different from wild-type at two mutational sites. We thus conclude that the evolutionary constraints imposed upon the protein, as it evolved from the state in which it existed in the primordial bacterium which gave rise to *Escherichia* and *Serratia,* permitted only those amino acid changes which maintained the integrity of the active site and of the configuration necessary for dimerization.

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[1] Yanofsky, C., D. R. Helinski, and B. D. Maling, *Cellular Regulatory Mechanisms,* Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 11.

[2] Garen, A., C. Levinthal, and F. Rothman, *J. Chem. Phys.,* **58,** 1068 (1961).

[3] Anfinsen, C. B., E. Haber, M. Sela, and F. H. White, Jr., these PROCEEDINGS, **47,** 1309 (1961).

[4] Baglioni, C., and V. M. Ingram, *Nature,* **189,** 465 (1961).

[5] Garen, A., and C. Levinthal, *Biochim. Biophys. Acta,* **38,** 470 (1960).

[6] Rothman, F., and R. Byrne, in press.

[7] Plocke, D., C. Levinthal, and B. L. Vallee, *Biochemistry,* in press.

[8] Hede, R., and C. Levinthal, unpublished results; Schwartz, J. H., and F. Lipmann, these PROCEEDINGS, **47,** 1996 (1961).

[9] Malamy, M., and B. S. Horecker, *Biochim. Biophys. Res. Comm.,* **5,** 104 (1961).

[10] Smithies, O., *Biochem. J.,* **61,** 629 (1955).

[11] Gomori, G., *J. Lab. Clin. Med.,* **37,** 570 (1951).

[12] Bach, M. L., E. R. Signer, C. Levinthal, and I. W. Sizer, *Fed. Proc.,* **20,** 255 (1961).

[13] "Multiple Molecular Forms of Enzymes," *Ann. N. Y. Acad. Sci.,* **94,** Art. 3 (1961).

[14] Spirin, A. S., A. N. Belozersky, N. V. Shugaeva, and B. F. Vanjushin, *Biokhimiya,* **22,** 744 (1957).

[15] Signer, E. R., A. Torriani, and C. Levinthal, *Cellular Regulatory Mechanisms,* Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 31.

[16] Torriani, A., and C. Levinthal, unpublished results.

[17] Schlesinger, M. J., unpublished results.

[18] Fincham, J. R. S., in *Advances in Enzymology and Related Subjects of Biochemistry,* ed. F. F. Nord (New York: Interscience, 1960), vol. 22, p. 1.

[19] Brenner, S., in *Symposium on Biochemistry of Human Genetics,* Ciba Foundation and International Union of Biological Sciences, ed. G. E. W. Wolstenholme and C. M. O'Connor (London: Churchill, 1959), p. 304.