utes. All measurements were replicated.

Within species, we sought variability for CO_2 compensation concentrations among 100 genetic lines of wheat *Triticum aestivum* L. and 20 genetic lines of barley *Hordeum vulgare* L. of diverse origin selected from the U.S. Department of Agriculture World Collections. Remarkably little variation in CO_2 compensation was found within these species, the compensation concentration being 52 ± 2 ppm (standard deviation) for wheat and 55 ± 2 ppm for barley.

Among the more than 300 monocotyledonous and 100 dicotyledonous species which we surveyed, CO_2 compensations were the same for all species within a genus except in three genera—*Euphorbia, Panicum,* and *Cyperus*—where we found both species with high and low compensation points in the same genus (Table 1). The *Panicum* species were of diverse origin and no single author has listed all of them in taxonomic groupings. In all cases, however, the phenotypes of species with high and low compensation points differed and, where subgenera classifications could be found, were grouped in separate subgenera.

In *Euphorbia,* only species of the subgenus Chamaesyce Raf. had low CO_2 compensation concentrations. In the *Cyperus* genus, *C. esculentus* L. of the subgenus Pycreus (Beav) C. B. Clarke had a low compensation point. *C. papyrus* L. had a high compensation point. The latter species is not listed by Fernald (*6*) but its form is greatly different from *C. esculentus.*

High and low CO_2 compensation points occur in different species of *Atriplex* (*7*). There, also, the traits were found in different subgenera. The recent report of Downton *et al.* (*8*) agrees with our observations within the *Panicum* genus. The fact that high and low CO_2 compensation points occur only in different subgenera indicates, perhaps, that the subgenera should be considered distinct genera. These contrasting compensation species do have many similar traits, however, and some of them may have sufficient genetic compatibility to permit viable crosses between them. Such crosses would provide unique material for study of the inheritance of basic physiological processes which are important in yielding ability of crop plants.

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References and Notes

1. D. N. Moss, *Nature* **193**, 587 (1962); W. J. S. Downton and E. B. Tregunna, *Can. J. Bot.* **46**, 207 (1968).
2. D. N. Moss, *Crop Sci.* **6**, 351 (1966).
3. E. B. Tregunna, G. Krotkov, C. D. Nelson, *Physiol. Plant.* **19**, 723 (1966); R. W. Downes and J. D. Hesketh, *Planta* **78**, 79 (1968).
4. H. P. Kortschak, C. E. Hartt, G. O. Burr, *Plant Physiol.* **40**, 209 (1965); M. D. Hatch, C. R. Slack, H. S. Johnson, *Biochem. J.* **102**, 417 (1967).
5. M. M. Rhoades and A. Carvalho, *Bull. Torrey Bot. Club* **71**, 335 (1944).
6. M. L. Fernald, *Gray's Manual of Botany* (American Book, New York, ed. 8, 1950).
7. E. B. Tregunna and J. Downton, *Can. J. Bot.* **45**, 2385 (1967).
8. J. Downton, J. Berry, E. B. Tregunna, *Science* **163**, 78 (1969).
9. A. S. Hitchbook, *Manual of the Grasses of the United States* (USDA Misc. Pub. No. 200, 1951).
10. Minnesota Agricultural Experiment Station Scientific Journal Series paper No. 6726. *Panicum* seeds supplied by the U.S. Department of Agriculture Southern Regional Plant Introduction Station.

27 November 1968; revised 6 February 1969

Immunological Detection of Single Amino Acid Substitutions in Alkaline Phosphatase

Abstract. *Antiserums to wild-type alkaline phosphatase from* Escherichia coli *were prepared and tested for reactivity with phosphatases altered by point mutations. Eight out of the nine mutant enzymes were distinguished from the wild type with quantitative microcomplement fixation. The structural changes are among the smallest yet observed immunologically.*

Certain immunological techniques can distinguish between proteins differing in primary sequence by a very few amino acids (*1*), or even one (*2*). To obtain further information about the value of such techniques, we have determined the immunological effects of single amino acid exchanges in *Escherichia coli* alkaline phosphatase. This dimeric protein has approximately 400 amino acid residues per polypeptide chain (*3*). Our discrimination between mutant and wild-type alkaline phosphatase marks the smallest relative change in protein primary structure observed immunologically to date.

Wild-type *E. coli* alkaline phosphatase was purified from a constitutive strain (C90F1) according to the procedure of Malamy and Horecker (*4*). Chromatographically purified alkaline phosphatase was also obtained from Worthington Biochemical Corporation. Enzymatic activity was assayed by rate of hydrolysis of *p*-nitrophenyl phosphate (*5*). Both protein preparations were emulsified in Freund's complete adjuvant supplemented with 4 mg of BCG (bacillus Calmette-Guérin) per milliliter and injected intradermally at four sites on the backs of male New Zealand white rabbits. Each rabbit received 0.7 to 0.8 mg of protein. Bleedings were taken at weekly intervals, beginning 2 weeks after the initial injection. After 11 weeks, each rabbit was given a course of three intravenous injections (1 mg each) on alternate days. Serums were collected 4 and 7 days after the last intravenous injection and stored in the frozen state. Three rabbits gave serums which reacted with only a single component in crude extracts of *E. coli,* when examined by Ouchterlony double diffusion, immunoelectrophoresis, and microcomplement fixation. Diffusion methods indicated that the fourth rabbit produced a low concentration of antibodies to a second component in crude extracts. This did not interfere with the microcomplement fixation analysis presented below. Most antiserums differed in titer, but not in discriminating capacity, and results below are averages from experiments carried out with many antiserums.

Strains of *E. coli* which constitutively produced mutant alkaline phosphatases were isolated as phosphatase-positive revertants of nonsense mutations, mapping in the alkaline phosphatase structural gene (*6*). The mutant enzymes were altered in their electrophoretic mobility, but they had regained almost full enzymatic activity. The mutations occurred at five sites. The nature of the amino acid substitutions at four of the sites was deduced from the electrophoretic changes (*7*). Five revertants were from the nonsense mutation at a fifth site. The substitutions at this site were determined by amino acid analyses of tryptic peptides (*8*).

No differences were detected between the mutants and the wild type by the double-diffusion technique. A spur was seen in the comparison of the phosphatases of *E. coli* and *Serratia marcescens,* which indicates that the two enzymes share only a limited number of antigenic determinants. The *Serratia* en-

Table 1. Mean complement fixation (CF) differences between wild-type and mutant alkaline phosphatases. The sites in the phosphatase gene at which the mutations occurred are arbitrarily designated *a* through *e*. All strains are from Garen's C90F series. The phosphatases of strains 272, 447, 722, 738, and 777 were tested with eight antiserums. The remaining strains were examined with one antiserum. The number of measurements on each strain was: 272 (15); 447 (19); 722 (34); 738 (16); 776 (4); 777 (20); 784 (4); 790 (4); 874 (15).

Strain	Amino acid replacement	Site	CF difference (%)	P*
1	Wild type		0	
272	Lysine→neutral	a	−1.2	<.5
447	Lysine→neutral	b	6.7	<.001
722	Glutamate→neutral	c	9.6	<.001
738	Lysine→neutral	d	6.6	<.001
776	Lysine→lysine†	e	−1.8	<.4
777	Lysine→glutamine	e	6.6	<.001
784	Lysine→tyrosine	e	10.0	<.05
790	Lysine→serine	e	9.5	<.01
874	Lysine→glutamate	e	17.1	<.001

* Probability that the complement fixation difference is zero (*11*). † A revertant to the wild type.

zyme differs greatly in primary structure from that of *E. coli* (*9*).

Wild-type and mutant enzymes were next compared by quantitative microcomplement fixation (Fig. 1); this test is very sensitive to small differences in protein structure (*10*). At the peak, 76 percent of the available complement was fixed by the wild-type enzyme, while only 67 percent was fixed by the mutant 874. This 9 percent difference in reactivity is referred to as the complement fixation difference. Experiments like this were conducted for each mutant, and the results are summarized in Table 1. Although all eight of the mutant enzymes reacted strongly with the antiserums, seven of them were significantly (*11*) less reactive than the wild type. The complement fixation differences were generally about 10 percent. These differences are small, compared to that between the wild-type enzyme and that of *Serratia*. Under these experimental conditions, the *Serratia* enzyme gave no reaction with our antiserums (*12*).

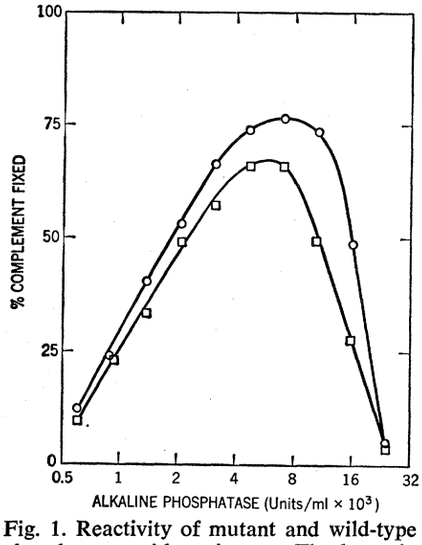

Fig. 1. Reactivity of mutant and wild-type phosphatases with antiserum. The bacteria were grown aerobically at 37°C in Difco nutrient broth, and alkaline phosphatase was extracted by heat shock (*16*). Serial dilutions of extract were tested for reactivity with 1-ml portions of a 1:10,000 dilution of antiserum in 7-ml reaction volumes containing a buffer described previously (*10*). The extracts were from strains C90F1 (wild type, ○) and C90F 874 (mutant, □). Pure enzyme from strain C90F1 produces a complement fixation curve identical to that of the crude extract.

In addition, one alkaline phosphatase mutant with specific activity reduced to less than 0.4 percent of wild type was examined. The point mutation in the genome of the strain, U13, destabilizes the normal dimeric form of the molecule (*13*). In contrast to the mutants studied above, the electrophoretic mobility of this mutant protein is identical with that of the wild-type enzyme (*13a*). The average complement fixation difference was 16 percent for this protein (*14*). Thus, the immunological reactivity of this mutant protein is strong, but significantly less than in those that retain enzymic activity. It appears that certain mutations may profoundly affect a protein's function without greatly altering its antigenic structure.

In summary, the majority of mutant alkaline phosphatases were distinguishable from the wild type by microcomplement fixation. All but one of these mutations involved changes in electrical charge; substitutions not involving differently charged amino acids have been detected immunologically in other proteins (*2*). No small alteration in primary sequence, even one reducing enzymatic activity, resulted in a large change in immunological reactivity. As all the discriminations were nearly as fine as this method permits, it is anticipated that single amino acid changes in larger proteins might not be detectable at all. From our data and that available (*2, 10*), it appears that microcomplement fixation can provide a sensitive measure of structural relationships between proteins (*15*).

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References and Notes

1. *Ribonuclease*: R. K. Brown, B. C. Tacey, C. B. Anfinsen, Jr., *Biochim. Biophys. Acta* **39**, 528 (1960); *Bird Lysozyme*: N. Arnheim, E. M. Prager, A. C. Wilson, *J. Biol. Chem.*, in press; *Phage Lysozyme*: M. Inouye and A. Tsugita, *J. Mol. Biol.* **37**, 213 (1968).
2. *Hemoglobin*: M. Reichlin, M. Hay, L. Levine, *Immunochemistry* **1**, 21 (1964); M. Reichlin, E. Bucci, C. Fronticelli, J. Wyman, E. Antonini, C. Ioppolo, A. Rossi-Fanelli, *J. Mol. Biol.* **17**, 18 (1966); *Coat Protein of Tobacco Mosaic Virus*: P. V. Sengbusch, *Z. Vererbungsl.* **96**, 364 (1965); M. H. V. Van Regenmortel, *Virology* **31**, 467 (1967); *Cytochrome c*: E. Margoliash, M. Reichlin, A. Nisonoff, in *Conformation of Biopolymers*, G. N. Ramachandran, Ed. (Academic Press, New York, 1967), vol. 1, p. 253; *β-Lactoglobulin*: N. I. Phillips, R. Jenness, E. B. Kalan, *J. Immunol.* **100**, 307 (1968); *Coat Protein of RNA Coliphage*: D. W. Scott, *Virology* **26**, 85 (1965); K. Weber and W. Konigsberg, *J. Biol. Chem.* **242**, 3563 (1967); K. Weber, *Biochemistry* **6**, 3144 (1967); J.-Y. Lin, C. M. Tsung, H. Fraenkel-Conrat, *J. Mol. Biol.* **24**, 1 (1967); A. C. Wilson and R. C. Valentine, unpublished results.
3. F. Rothman and R. Byrne, *J. Mol. Biol.* **6**, 330 (1963).
4. M. H. Malamy and B. L. Horecker, *Biochemistry* **3**, 1893 (1964).
5. N. Arnheim, Jr., G. T. Cocks, A. C. Wilson, *Science* **157**, 568 (1967).
6. Strains provided by Dr. Alan Garen.
7. A. Garen, personal communication.
8. M. G. Weigert, E. Lanka, A. Garen, *J. Mol. Biol.* **23**, 391 (1967).
9. E. R. Signer, A. Torriani, C. Levinthal, *Cold Spring Harbor Symp. Quant. Biol.* **26**, 31 (1961).
10. N. Arnheim, Jr., and A. C. Wilson, *J. Biol. Chem.* **242**, 3951 (1967); V. M. Sarich and A. C. Wilson, *Science* **154**, 1563 (1966); L. Levine and H. Van Vunakis, *Meth. Enzymol.* **11**, 928 (1967).
11. Determined by *t*-test applied to paired comparisons of mutant and wild-type phosphatases [G. G. Simpson, A. Roe, R. C. Lewontin, *Quantitative Zoology* (Harcourt Brace, New York, 1960), p. 180].
12. A reaction was observed with the *Serratia* enzyme when the antiserum concentration was raised by a factor of about 25.
13. M. J. Schlesinger, A. Torriani, C. Levinthal, *Cold Spring Harbor Symp. Quant. Biol.* **28**, 539 (1963).
13a. M. J. Schlessinger and C. Levinthal, *J. Mol. Biol.* **7**, 1 (1963).
14. The magnitude of the difference between this antigen and the wild type was not the same with all the antiserums. One full course serum gave a mean complement fixation difference (26 percent) greater than that measured by other serums (10 percent).
15. T. M. Murphy and S. E. Mills [*Arch. Biochem. Biophys.* **127**, 7 (1968)] report that several single and double amino acid mutations in the α-subunit of *E. coli* tryptophan synthetase cannot be detected by microcomplement fixation. The reasons for the differences between these results and our own are not clear. Our larger number of paired comparisons of mutant and wild type may give statistical significance to small differences.
16. A. Garen and C. Levinthal, *Biochim. Biophys. Acta* **38**, 470 (1960).
17. Supported by an NSF grant to A.C.W. and an NSF predoctoral fellowship to G.T.C. We thank Alan Garen for discussion and for the bacterial strains, and C. L. Chiang for help with the statistics.

16 December 1968