TRANSFERRIN POLYMORPHISM IN PIGEONS

Thesis by

Jeffrey A. Frelinger

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1973

(submitted December 13, 1972)

FOR JOY

ACKNOWLEDGMENTS

I thank Ray Owen for his help and counsel during my stay at Caltech. His insight and knowledge have provided a continuing model for me. I thank Lee Hood and Paul Hargrave for helping me learn protein chemistry. I thank the other members of my committee Ed Lewis, Herschel Mitchell, and Dick Russell for their interest in my work.

I am grateful to Ron Acton who showed me Science can be a business, and Dave Dusenbery who showed me it was fun. I thank my fellow students, Tom Douglas, Sue Melvin, and Su Rosenberg for tolerating me, especially during the composition of this thesis. I also thank all the other people who made this work possible, Frank, Richard and Dale in the machine shop, Ed, Bill and Jane in the stockroom, Milton and the animal room crew.

I thank the National Institutes of Health and Caltech for financial support.

Finally, I thank my friends, Carol, Jim, Bill, Janet, Lance, Marianne, Sue and all the others who made Caltech a human experience.

ABSTRACT

Transferrin polymorphism, detected by differences in electrophoretic mobility among allelic forms, is widespread in vertebrates. In pigeons, two alleles have been reported. In a developmental study, I have shown that for a period following hatching the young bird's transferrin phenotype reflects the maternal rather than its own genotype, although the squab is actively synthesizing transferrin in its liver. This probably reflects transfer of maternally derived protein through the egg. As the period of maternal transfer corresponds to the period of immunoincompetence on the part of the squab, the transferrin is known to be bacterio-and fungistatic, I investigated the possibility of differences in the funistatic effects of the three transferrin phenotypes, using yeast as an assay organism. I found that transferrin derived from heterozygotes is much more effective in the inhibition of yeast growth than that from homozygotes. Under the breeding conditions in our flock, embryonic mortality was significantly lower among the progeny of heterozygous females. This suggests that transferrin polymorphism is maintained by selection against the progeny of homozygous females. An algebraic consideration of this form of selection leads to the prediction that a population at equilibrium for allele frequencies would be in Hardy-Weinberg equilibrium for phenotype frequencies. The published data of others, in addition to my own, show that real pigeon

populations are in Hardy-Weinberg equilibrium.

Transferrin was purified (> 95% pure) from all three phenotypes; all had similar amino acid compositions and molecular weights. Peptide mapping of the allele products revealed a difference in one peptide, which on analysis was consistent with a single amino acid substitution (Ser → Asp). Mixtures of homozygous type transferrins, while appearing electrophoretically identical to the heterozygous type, do not equal its behavior in yeast inhibition. This suggests the presence of "hybrid molecules" synthesized in heterozygotes. I found that pigeon transferrins can be dissociated to 40,000 M.W. fragments by heating in sodium dodecyl sulfate (SDS). Removal of SDS results in reassociation into 80,000 M.W. dimers. All preparations show only a single amino terminal (alanine). CNBr cleavage produces only four fragments in spite of the fact that there are eight methionines. Sequence data may indicate multiple amino acid sequences. Quantitative hydrozanalysis yields only a single carboxyl terminal (serine). These data suggest that pigeon transferrin may be a dimer.

TABLE OF CONTENTS

CHAPTER	TITLE	PAGE
	Dedication---	ii
	Acknowledgments--------------------------------------	iii
	Abstract---	iv
	GENERAL INTRODUCTION---------------------------------	1
I	Maternally Derived Transferrin in Pigeon Squabs------	17
	Additional Data--------------------------------------	20
	Reference--	21
II	Maintenance of Transferrin Polymorphism in Pigeons---	24
III	Transferrin Polymorphism and the Hardy-Weinberg Ratios--	29
IV	Chemical Basis of Transferrin Polymorphism in Pigeons---	39
V	The Covalent Structure of Transferrins---------------	52
VI	Summary and Recapitulation---------------------------	81

INTRODUCTION

Transferrin is a large, non-heme, iron binding protein, which binds two moles of iron per mole of protein. Since its initial description in 1951 (1,2), it has been investigated by physical biochemists interested in its iron binding properties (3-17); by protein chemists interested in its covalent structure and the in the number of polypeptides per mole of intact protein (18-50); by physiologists to discover its role in iron transport and control of erythropoesis (51-66); and by geneticists who have been intrigued by its widespread polymorphism (57-116). There have been few attempts to correlate the studies conducted in different fields. Most of the experiments were performed using human serum transferrin, chicken egg white or rabbit serum transferrin; little work has been done on protein derived from other species.

The chemical studies of transferrin have centered on investigation of the iron binding site, carried out principally by spectroscopy. It has now been firmaly established that the iron binding occurs to three ionized tyrosine residues and a single bicarbonate ion, with some involvement of nitrogen ligands (3,7,12,13,16,17), in tetrahedral bonding to the iron. The mechanism (for review see 117) is highly specific for iron, the preference being several magnitudes higher for iron III than for any other oxidation state of iron or other metal ions except Cr^{3+} (2). The order obtained

is precisely what would be expected when charge density and size are considered (117).

Earlier work (4) had suggested that the two iron binding sites were equivalent in each molecule. Recently Luk (11) has proposed, on the basis of fluorescence spectroscopy, that the iron binding sites are far enough apart to be non-interacting, but are not equivalent, as only one mole of Pr^{3+} or Nd^{3+} is bound per mole of protein. These data, based on lanthanide binding, may be interpreted differently, as due to severe allosteric effects caused by the large size of the Pr^{3+} or Nd^{3+} ions which might destroy the conformation of the second metal binding site. The distinction between identical and equivalent must be very carefully made. The two sites are not equivalent for lanthanide binding, but they could easily be equivalent for binding Fe^{3+}, a much smaller ion. At present, therefore, the equivalence of the iron binding sites must be considered open to doubt.

The covalent structure of transferrin has been extensively studied in the protein obtained from chicken egg white and that from human serum (30,34). On the basis of several lines of evidence, one would expect transferrin to be a dimeric molecule. It binds two moles of iron/mole or protein, has fewer than the expected number of tryptic peptides, and its molecular weight of 80,000 is high for a single polypeptide chain. Jeppsson (32) reported a possible dimeric structure for human serum transferrin but this was

not subsequently confirmed by others (30,34). A single polypeptide chain model has been reported by Feeney (30) for chicken transferrin. Mann *et al*. (34) have proposed a similar model for human serum transferrin. It has recently been suggested that the transferrin structural gene was formed by a fused tandem duplication (41). This is based on the analysis of cyanogen bromide fragments of chicken ovotransferrin (41), in which molecular weight and charge similarities of the pairs of the fragments suggested some duplicate structure.

In two other species, turtles (36) and cattle (26), evidence for subunit structure has been found by ultracentrifugation. Hagfish transferrin has been reported to have a molecular weight of only 40,000, approximately one-half the common weight (36). However, Aisen *et al*. (18) attempting to repeat this work, reported a molecular weight of 80,000 for hagfish transferrin.

Mammalian milk also contains an iron binding protein (118). This protein, lactoferrin, was originally thought to be related to transferrin. The issue was confused by proof of identity of a serum and a milk iron binding protein in rabbits (21,53). The current belief is that there are at least two distinct iron binding proteins in milk, transferrin and lactoferrin, whose ratios may vary among species. In rabbits the milk iron binding protein is almost exclusively transferrin (21,53); in humans it is lactoferrin (119, 120, 121). In cattle, milk contains a mixture of the two proteins

(118, 122). In pigeon milk, the only iron binding protein which has been identified is transferrin (this thesis).

The role of transferrin in erythropoeisis is well established. Iron-saturated transferrin functions as an iron donor for hemoglobin synthesizing reticulocytes (51,52,61,63). *In vitro* this process requires citrate or other iron chelates as cofactors (5). Transferrin preferentially binds to reticulocytes rather than to erythrocytes. Iron-saturated transferrin is more likely than iron free transferrin to bind to the reticulocytes.

Transferrin is synthesized primarily in the liver of mammals (123), although its synthesis can also be detected in leukocytes (139). In birds, the oviduct also synthesizes ovotransferrin (104).

Susceptibility to systemic fungal infection, particularly candidasis, has been associated with decreased serum transferrin levels. This is particularly important in patients who are immuno-suppressed, either intentionally, as in the case of kidney transplant recipients, or unintentionally as in the case of leukemic patients (126). The mechanism of the antifungal activity of serum transferrin has been of interest for many years. The first obervation of the bacteriostatic properties of transferrin predated modern description of the protein in physical-chemical terms (127). It has become clear that transferrin functions by binding tightly to the available iron (128-131), thus making iron unavailable for use by the microorganism. Many organisms are iron-dependent or need iron for toxin production

(132), and can be effectively inhibited by this mechanism. This appears to be the mode of action of transferrin on microbes *in vitro*.

Iron is known to have contraceptive properties (133). Transferrin has been shown to be present in seminal fluid (134, 135), and a related protein, lactoferrin, is found in vaginal secretions (136). The obvious mechanism seems to be that iron saturates the transferrin and allows local infection to take place. This provides an environment not healthy for either the sperm or the eggs, and their decreased viability and shorter functional life decreases the probability of fertilization as well as survival of the embryos.

Transferrin has been found to be polymorphic in most vertebrate species examined. The only exceptions seem to be those which have recently gone through a population bottleneck, such as the American Bison (137). Polymorphic transferrins are usually differentiated by differences in electrophoretic mobility on either starch or acrylamide gel. Usually, one particular allele is frequent and there are many low-frequency variants. At least eighteen alleles are known in man (138), many represented only by a single family. Some alleles, however, are common in some populations but rare in others. C transferrin is the most common allele but transferrin D is common in negro populations and D^{chi} in oriental and some American Indian populations (67,138).

In pigeons, on the other hand, there are only two known alleles,

first reported by Mueller *et al*. (101). The allelic transferrins differ in electrophoretic mobility on starch gels. The two alleles have been reported at nearly equal frequency in such diverse places as California, Wisconsin, Missouri, and North Ireland (78, 81, 101). In pigeons as in other species studied, transferrin appears to be inherited as an autosomal codominant trait. This is based on the observation that artificial mixtures of homozygous transferrins appear identical to heterozygous material on electrophoresis. Ashton has suggested that transferrin polymorphism in cattle may be maintained by heterozygote superiority. He reported excess numbers of heterozygotes in some populations, and in the progeny of some mating combinations (68, 69, 72). Subsequent study of other breeds of cattle (70) has failed to confirm Ashton's conclusions. In mice, conflicting reports on the effects of transferrin type on fertility have appeared (71, 102). Some workers have found no relationship (102), while others have shown small but statistically significant effects among some combinations of genotypes (71). These data have been used to explain the absence of transferrin A in wild mouse populations. In vole populations, the disadvantage of one of the alleles has been indicated by field studies, but no evidence of heterozygote superiority has been uncovered, nor a mechanism of selection proposed (85,86,110). In swine, conflicting findings have been reported on transferrin effects on fertility (83,93).

The study of pigeon transferrin polymorphism reported in this thesis is the only one to report a systematic consideration from the population to the molecular levels.

REFERENCES

1. Koechlin, B.: J. Am. Chem. Soc.: 74:2649 (1952)
2. Warner, R. C.; Weber, I.: J. Biol. Chem. 191:173 (1951)
3. Aasa, R.; Aisen, P.: J. Biol. Chem. 243:2399 (1968)
4. Aasa, R.; Malmstrom, B. G.; Saltman, P.; Vanngard, T.: Biochim. Biophys. Acta 75:203 (1963)
5. Aisen, P.; Leibman, A.: Biochem. Biophys. Res. Comm. 32:220 (1968)
6. Aisen, P.: Biochem. Biophys. Res. Comm. 30:407 (1968)
7. Azari, P.; Phillips, J. L.; Arch. Biochem. Biophys. 138:32 (1970)
8. Bezkorovainy, A.: Biochim. Biophys. Acta. 127:535 (1966)
9. Davis, P.; Saltman, P.; Benson, S.: Biochim. Biophys. Res. Comm. 8:56 (1962)
10. Lehrer, S. S.: J. Biol. Chem. 244:3613 (1969)
11. Luk, C. K.: Biochem. 10:2838 (1971)
12. Komatsu, S. K.; Feeney, R. E.: Biochem. 6:1136 (1967)
13. Phillips, J. L.; Azari, P.: Arch. Biochem. Biophys. 151:445 (1972)
14. Warner, R. C.; Weber, I.: J. Am. Chem. Soc. 75:5094 (1953)
15. Williams, J.; Phelps, C. F.; Lowe, J. M.: Nature 226:858 (1970)
16. Zschocke, R. H.; Chiao, M. T.; Bezkorovainy, A.: Eur. J. Biochem. 27:145 (1972)
17. Warner, R.C.: Trans. N. Y. Acad. Sci. 16:182 (1953)
18. Aisen, P.; Liebman A.; Sia, C.: Biochem. 11:3461 (1972)
19. Aisen, P.; Koenig, S. H.; Schillinger, W. E.; Scheinberg, I. H.; Mann, K. G.; Fish, W.: Nature 226:859 (1970)

20. Allison, R. G.; Feeney, R. E.: Arch. Biochem. Biophys. 124:548 (1968)

21. Baker, E.; Shaw, D. C.; Morgan, E. H.: Biochem. 7:1371 (1968)

22. Bezkorovainy, A.; Grohlich, D.; Gerbeck, C.: Biochem. J. 110: 765 (1968)

23. Bezkorovainy, A.; Rafelson, M.; Likttite, A.: Arch. Biochem. Biophys. 103:371 (1963)

24. Boffa, G. A.; Drilhou, A.; Favre, A.; Zakin, M. M.; Fine, J. M.; C. R. Acad. Sci. Serus D 267:1067 (1968)

25. Clark, J.; Osuga, D.; Feeney, R. E.: J. Biol. Chem. 238:3621 (1963)

26. Efremov, G. D.; Smith, L. L.; Barton, B. P.; Huisman, T. H. J.: Animal Blood Groups, Biochem. Genet. 2:159 (1971)

27. Elleman, T. C.; Williams, J.: Biochem. J. 116:515 (1970)

28. Ezekiel, E.; Lai, L. Y. C.; Kalder, E.: Comp. Biochem. Physiol. 10:69 (1963)

29. Frenoy, N.; Groussault, Y.; Bourrillon, R.: Biochimie 53: 1207 (1971)

30. Greene, F. C.; Feeney, R. E.: Biochem. 7:1366 (1968)

31. Jamieson, G. A.; Jett, M.; DeBernardo, S. L.: J. Biol. Chem. 246:3686 (1971)

32. Jeppsson, J. O.: Acta Chim. Scand. 21:1686(1967)

33. Jordan, S. M.; Morgan, E. H.: Comp. Biochem. Physiol. 29: 383 (1969)

34. Mann, K. G.; Fish, W.; Cox, A. C.; Tanford, C.: Biochem. 9:1348 (1970)

35. Osuga, D.; Feeney, R. E.: Arch. Biochem. Biophys. 124:560 (1968)

36. Palmour, R. M.; Sulton, H. E.: Biochem. 10:4026 (1971)

37. Parker, W. C.; Bearn, A. G.: Science 133:1014 (1961)

38. Parker, W. C.; Bearn, A. G.: J. Exp. Med. 115:83 (1962)

39. Parker, W. C.; Hagstrom, J. W. C.; Bearn, A. G.: J. Exp. Med. 118:975 (1963)

40. Patras, B.; Stone, W. H.: Proc. Soc. Exp. Biol. Med. 107:118 (1961)

41. Phillips, J. L.; Azari, P.: Biochem. 110:1160 (1971)

42. Robinson, J. C.; Pierce, J. E.: Arch. Biochem. Biophys. 106: 348 (1964)

43. Rosseneu-Motreff, M. Y.; Soetewey, F.; Lamote, R.; Peters, H.: Biopolymers 10:1039 (1971)

44. Stratil, A.; Spooner, R. L.: Biochem. Genet. 5:347 (1971)

45. van Eijk, H.G.; van Dijk, J. P.; van Noort, W. L.; Leijnse, B.; Monfoort, C. H.: Scand. J. Haem. 9:267 (1972)

46. Webster, R. O.; Pollara, B.: Comp. Biochem. Physiol. 30:509 (1969)

47. Williams, J.: Biochem. J. 83:355 (1962)

48. Williams, J.: Biochem. J. 108:57 (1968)

49. Williams, J.; Wenn, R. V.: Biochem. J. 116:533 (1970)

50. Zschoke, R. H.; Bezkorovainy, A.: Biochem. Biophys. Acta. 200:241 (1970)

51. Baker, E.; Morgan, E. H.: Biochem. 8:2954 (1969)

52. Baker, E.; Morgan, E. H.: Biochem. 8:1133 (1969)

53. Baker, E.; Jordan, S.; Toffery, A.; Morgan, E. H.: Life Sci. 8:89 (1969)

54. Baker, E.; Morgan, E. H.: Quart. J. Exp. Physiol. 54:173 (1969)

55. Douglas, T. A.; Renton, J. P.; Watts, C.: Brit. J. Haematology 20:185 (1971)

56. Douglas, T. A.; Renton, J. P.; Wright, R.: Am. J. Obst. Gyn. 102:1169 (1968)

57. Fielding, J.: Edwards, S.; Ryall, R.: J. Clin. Path. 22:677 (1969)

58. Fletcher, J.: Clin. Sci. 37:273 (1968)

59. Fletcher, J.; Huehns, E. R.: Nature 218:1211 (1968)

60. Kornfeild, S.: Biochim. Biophys. Acta. 194:25 (1969)

61. Martinez-Medellin, J.; Schulman, H. M.: Biochim. Biophys. Acta. 264:272 (1972)

62. Morgan, E. H.; Baker, E.: Biochim. Biophys. Acta. 184:442 (1969)

63. Morgan, E. H.: Biochim. Biophys. Acta 244:103 (1971)

64. Schade, S.; Bernier, G.; Conrad, M.: Brit. J. Haematol. 17: 187 (1969)

65. Tarvydas, H.; Jordan, S.; Morgan, E. H.: Brit. J. Nutrit. 22:565 (1968)

66. Tojvanen, P.; Ross, T.; Hirvonen, T.: Scand. J. Haematol. 6:113 (1969)

67. Arends, T.; Gallango, M. L.: Science 143:367 (1964)

68. Ashton, G. C.: Nature 183:404 (1958)

69. Ashton, G. C.: Genetics 52:983 (1965)

70. Ashton, G. C.; Lampkin, G. H.: Genet. Res. 6:209 (1965)

71. Ashton, G. C.; Dennis, M. N.: Genetics 67:253 (1971)

72. Ashton, G. C.; Hewetson, R. W.: Animal Prod. 11:533 (1969)

73. Baker, C.M.A.; Croizier, G.; Stratil, A.; Manwell, C.: Advances in Genetics 15:147 (1970)

74. Baker, C. M. A.: Genetics 58:211 (1968)

75. Baker, C. M. A.: Comp. Biochem. Physiol. 20:949 (1967)

76. Baker, C. M. A.; Manwell, C.; Labisky, R. F.; Harper, J. A.: Comp. Biochem. Physiol. 17:467 (1966)

77. Baker, C. M. A.; Hanson, H. C.: Comp. Biochem. Physiol. 17: 997 (1969)

78. Brown, R. V.; Sharp, H. B. Jr.: Animal Blood Groups, Biochem. Genet. 1113 (1970)

79. Brush, A. H.: Comp. Biochem. Physiol. 25:159 (1968)

80. Chen, S. H.; Sutton, H. E.: Genetics 56:425 (1967)

81. Ferguson, A.: Comp. Biochem. Physiol. 38B:477 (1971)

82. Cohen, B. C.: Genet. Res. 1:431 (1960)

83. Fesus, L.; Rasmussen, B. A.: Animal Blood Groups, Biochem. Genet. 257 (1971)

84. Fowle, K. E.; Cline, J. H.; Klosterman, E. W.; Parker, C. F.: J. Animal Sci. 26:1226 (1967)

85. Gaines, M. S.; Krebs, C. J.: Evolution 25:702 (1951)

86. Gaines, M. S.; Myers, J.; Krebs, C. J.: Evolution 25:443 (1971)

87. Giblett, E. R.; Hickman, C. G.; Smithies, O.: Nature 183:1589 (1959)

88. Gilmour, D. G.; Morton, J. R.: Theor. Appl. Genet. 41:47 (1971)

89. Hershberger, W. K.: Trans. Am. Fish Soc. 99:207 (1970)

90. Jamieson, A.: Heredity 20:419 (1965)

91. Jeppsson, J. O.: Biochim. Biophys. Acta 140:465 (1967)

92. Klein, P. A.; Roop, B. L.; Roop, W. E.: Nature 212:1376 (1966)

93. Kristjansson, F. K.: J. Reprod. Fertil. 8:311 (1964)

94. Kuzmenko, L. G.; Tsitol, Genet. 2:469 (1968)

95. Lai, L. Y. C.: Folia Primetologica 17:193 (1972)

96. Lush, I. E.: Nature 189:981 (1961)

97. Mao, S. H.; Dessquer, H. C.: Comp. Biochem. Physiol. 40A: 669 (1971)

98. McDermid, E. M.; Vos. G. H.: S. Afr. J. Med. Sci. 36:7 (1971)

99. McDougal, E. I.; Lowe, V. P. W.: J. Zool. Soc. Lond. 155:133 (1968)

100. Moriwaki, K.; Tsuchiya, K.; Yosida, T. H.: Genetics 63:193 (1968)

101. Mueller, J.; Smithies, O.; Irwin, M. R.: Genetics 47:1385 (1962)

102. Nagai, J.; Kristjansson, F. K.: Can. J. Genet. Cytol. 12:307 (1970)

103. Nadler, C.: Comp. Biochem. Physiol. 27:487 (1968)

104. Ogden, A. L.; Morton, J. R.; Gilmour, O. G.; McDermid, E. M. Nature 195:1026 (1962)

105. Roop, W.; Putnam, F.: J. Biol. Chem. 242:2507 (1967)

106. Shreffler, D. C.: Proc. Nat. Acad. Sci. (US) 46:1378 (1960)

107. Shreffler, D. C.: J. Heridity 54:127 (1961)

108. Spooner, R. L. and Baxter, G.: Biochem. Genet. 2:371 (1969)

109. Stratil, A.: Comp. Biochem. Physiol. 24:113 (1968)

110. Tamarin, R. H.; Krebs, C. J.: Evolution 23:183 (1969)

111. Vohs, P. A.; Carr, L. R.: Condor 71:413 (1969)

112. Wang, A. C.; Shustes, J.; Epstein, A.; Fudenberg, H. H.: Biochem Genet 1:347 (1968)

113. Wang, A. C.; Sutton, H. E.; Riggs, A.: Am. J. Hum. Genet. 18:454 (1966)

114. Watanabe, S.; Nozawa, K.; Suzuki, S.: Proc. Jap. Acad. 41: 326 (1965)

115. Watanabe, S.; Suzuki, S.: Proc. Jap. Acad. 42:178 (1966)

116. Wright, J. E.; Atherton, L. M.: Trans. Am. Fish Soc. 99: 179 (1970)

117. Feeney, R. E.; Komatsu, S. K.: Struct. Bonding 1:146 (1966)

118. Groves, M. L.: J. Am. Chem. Soc. 82:3345 (1960)

119. Blanc, B.; Isliker, H.: Bull. Soc. Chim. Biol. 43:929 (1961)

120. Masson, P. L.; Heremans, J. F.: Comp. Biochem. Physiol. 39B: 119 (1971)

121. Jorden, S. M.; Morgan, E. H.: Nature 215:76 (1967)

122. Groves, M. L.: Biochim. Biophys. Acta 100:154 (1965)

123. Goldsworthy, P. D.; McCarter, H. R.; McGuigan, J. E.; Peppers, G. F.; Volwiler, W.: Am. J. Physiol. 218:1428 (1970)

124. McFarlane, H.; Reddy, S.; Adcock, K. J.; Adeshina, H.; Cooke, A. R.; Akene, J.: Brit. Med. J. 4:268 (1970)

125. McFarlane, H.; Okubadejo, M.; Reddy, S.: Am. J. Clin. Path. 57:587 (1972)

126. Caroline, L.; Rosner, F.; Kozinn, P. J.: Blood 34:441 (1969)

127. Schade, A. L.; Caroline, L.: Science 100:14 (1944)

128. Feeney, R. E.: Arch. Biochem. Biophys. 34:196 (1951)

129. Feeney, R. E.; Nagy, D. A.: J. Bacteriol. 64:629 (1952)

130. Fraenkel-Conrat, H.; Feeney, R. E.: Arch. Biochem. 29:101 (1950)

131. Oran, J. D.; Reittler, B.: Biochim. Biophys. Acta 170:351 (1968)

132. Kochan, I.; Golden, C. A.; Bukovic, J. A.: J. Bacteriol. 100: 64 (1968)

133. Loewit, K.; Fodisch, H. J.; Zambelis, N.; Egg, D.: Contraception 6:65 (1972)

134. Roberts, T. K.; Boettcher, B.: J. Reprod. Fertil. 18:347 (1969)

135. Stratil, A.: Proc. 11th European Congr. Blood Groups and Protein Polymorphisms in Animals P. 417 (1968)

136. Masson, P. L.; Heremans, J. F.; Ferin, J.: Fertil. and Steril. 19:679 (1968)

137. Braend, M.; Stormont, C.: Nature 197:910 (1963)

138. Giblett, E. R.: Genetic Markers in Human Blood, F. A. Davis Company, Philadelphia (1969)

139. Soltys, H. D.; Brody, J. I.: J. Lab. Clin. Med. 75:250 (1970)

CHAPTER 1

Maternally Derived Transferrin in Pigeon Squabs

The publication contained in this chapter is from Science and is reproduced here with the permission of the American Association for the Advancement of Science.

Reprinted from
26 March 1971, Volume 171, Pages 1260-1261

SCIENCE

Maternally Derived Transferrin in Pigeon Squabs

Abstract. *With the use of genetically marked transferrin, a major portion of circulating transferrin from a newly hatched squab was found to be derived from the mother through the egg. The transfer is not through the parental crop milk. The squab does not accumulate enough transferrin of its own making to be detectable until it is about 8 days old. The maternally derived protein remains detectable until 14 days after hatching. The squab actively synthesizes a portion its own transferrin from hatching onward.*

Transferrin is a nonheme iron-binding protein (*1*) that is widely distributed throughout the chordates from lamprey to man (*2*). One of its principal functions is the transport of iron to reticulocytes for incorporation into hemoglobin (*3*). It may be important in nonspecific resistance to disease, as well as for its iron transport function. Transferrin has been found to be polymorphic in most species, the products of different alleles usually differing in electrophoretic mobility (*4*).

Although the development of transferrin has been studied in several mammalian species, including mouse, rabbit, and man (*5*), I am not aware of any reports on developmental studies of transferrin in birds. In mammals, there is very little transport of transferrin to the developing fetus (*6*); in this, transferrin differs from certain immunoglobulins, which are known to be transported intact to the embryo.

Pigeons have been shown to be polymorphic at a transferrin locus, and have only two reported variants, Tf^A and Tf^B (*7*). The alleles are inherited as autosomal codominants, and in populations so far examined they are present in nearly equal frequency. Transferrin is found in the pigeon blood, egg white, egg yolk, crop milk, and probably in the semen. In all these fluids it is under the control of the same gene. In pigeons, which differ in this respect from chickens (*8*), the electrophoretic mobility of transferrin in all these fluids is identical. In this report, therefore, transferrin derived from egg white will not be distinguished by the specific term ovotransferrin, which is often used.

Pigeons (*Columba livia*) were maintained in the laboratory in mating cages (1 pair per cage) or in large fly-pens. Eggs were checked daily and the day of hatching was designated as day 0.

For adults and squabs over 4 days old, when more than 0.5 ml of blood was to be removed, the birds were bled from the brachial vein by syringe and needle. The blood was allowed to clot and the serum was collected. For younger birds, blood was collected directly onto filter paper for electrophoresis. In order to determine whether this would give consistent serum patterns, several squabs were killed by exsanguination and their serums were collected. The results for collection by filter paper and by syringe were identical.

Transferrin typing was carried out on horizontal starch gels by the use of tris–citric acid buffer (*p*H 7.5) in the gel and borate buffer (*p*H 8.7) as electrolyte (*9*). An ice pack was placed on top of the gel to prevent excessive heating; the gels were stained with Coomassie blue.

Antibodies to pigeon transferrin were

prepared in rabbits. Approximately 5 mg of purified pigeon transferrin in Freund's complete adjuvant (Difco) was injected into toe pads. Four weeks later, 2 mg of this transferrin in 1 ml of saline was injected intraperitoneally; and 3 days after that, 3 mg in 1 ml of saline was injected intravenously. The rabbits were bled 1 week after the last injection. When assayed by immunoelectrophoresis against pigeon normal serum, the rabbit antiserum gave a single band in the transferrin position.

For determination of the synthesis of transferrin by newly hatched squabs, the squabs were injected intraperitoneally with [^{3}H]leucine (specific activity, 38.5 c/mmole; New England Nuclear) and allowed 20 minutes to incorporate the isotope. The squabs were killed; their livers were washed in ice-cold phosphate buffered saline (PBS) (*10*) and homogenized in a Waring Blendor for 2 minutes in 15 ml of PBS. The homogenate was centrifuged 1 hour at 30,000*g* and the pellet was discarded. A portion of the supernatant was precipitated with an equal volume of 10 percent trichloroacetic acid (TCA) and counted in a toluene omnifluor scintillation mixture. One-half milliliter of supernatant was reacted with 0.5 ml of antiserum for 1 hour at room temperature, and then for 2 days at 4°C. The pellets were washed four times in cold PBS, precipitated with TCA, filtered, and counted as above.

The following matings were made to test for maternally derived transferrin: Tf^A/Tf^A ♀ × Tf^B/Tf^B ♂; the reciprocal Tf^B/Tf^B ♀ × Tf^A/Tf^A ♂; and Tf^A/Tf^B ♀ × Tf^A/Tf^A ♂. In the AA ♀ × BB ♂ mating the squab is genotypically AB. However, until 8 days after hatching it appears to have only A type transferrin in its serum, as assayed on starch gels. On the eighth day, the squab's own type becomes detectable. A similar result is obtained in BB ♀ × AA ♂ matings, the offspring showing only B type until 8 days. In AB ♀ × BB ♂ matings, although half the squabs are homozygous BB, all show the heterozygous phenotype until they reach 14 to 16 days of age.

Because transferrin is found in crop milk produced by both the male and the female, protein transfer by this route could affect the results. One pair of squabs of AB genotype was separated at hatching; one squab was put with the A parent, the other with the B parent. Both squabs followed the same pattern; that is, the squabs showed the maternal phenotype regardless of the source of crop milk.

Table 1. Active synthesis of transferrin by squab liver. Phosphate buffered saline (0.5 ml) and 0.5 ml of liver homogenate were centrifuged at 30,000*g*. The supernatant and 0.5 ml of the indicated serums were reacted, pellets were washed and precipitated with TCA as described in the text. The values are given in counts per minute per filter and represent the average of duplicate portions of the same homogenate, subjected to the same treatments.

	Squab No. 1 (0 days, 17 g)	Squab No. 2 (0 days, 17 g)	Squab No. 3 (4 days, 70 g)
Saline	74	30	221
Rabbit normal serum	195	105	204
Rabbit antiserum to human serum	95		
Rabbit antiserum to human serum + human serum	87		
Rabbit antiserum to pigeon serum	3721	4483	1919
Rabbit antiserum to pigeon transferrin	1068	1152	884
Supernatant (0.5/ml)	5688	4027	3038

These experiments demonstrate the transfer of a maternally derived intact protein through the egg, or as a formal possibility, a portion of the maternal protein-synthesizing machinery. Because the homozygous offspring of matings of AB ♀ × AA ♂ show the heterozygous type even though they do not possess the Tf^B gene, the protein's source can only be maternal.

These observations necessitated a study of active synthesis of transferrin by squabs. We tested squabs at various times after hatching for their ability to incorporate [^{3}H]leucine into protein reacting with monospecific antiserums to transferrin. The results are given in Table 1. Antiserum to human serum, alone and with added human serum, served as coprecipitation controls; these controls are not different from the rabbit normal serum or saline controls. The antiserum to transferrin precipitates about 20 percent of the total labeled protein in the supernatant, while the antiserum to pigeon whole serum precipitates almost all of the counts.

With the use of genetically marked transferrin, this study has shown that, in pigeons, newly hatched squabs derive part of their plasma proteins from their mothers. The transferred protein represents the major portion of the young squab's serum transferrin, even though the young bird is actively synthesizing transferrin during this period. This conclusion is reasonable when the growth rate of a young pigeon is considered. The squab, weighing about 16 g at hatching, doubles its weight in the first 2 days, and by 2 weeks it weighs 150 g (*11*). This rapid rate of growth involves a large increase in mass, and extensive synthesis is required even to maintain a constant concentration of transferrin.

All the observations are consistent with the following model. A large amount of transferrin is absorbed intact by the developing embryo, through the egg white or the yolk, or both. Some time prior to the day of hatching, the squab initiates its own synthesis of transferrin. The failure to detect the squab's own type in genotypically heterozygous squabs is a result of inability to detect low levels of transferrin. Unlike mammals, therefore, pigeons provide the major part of their offspring's transferrin supply by maternal transfer, even though the squab is also engaged in active transferrin synthesis.

JEFFREY A. FRELINGER

Division of Biology, California Institute of Technology, Pasadena 91109

References and Notes

1. R. Feeney and S. Komatsu, *Struct. Bonding* 1, 146 (1966).
2. R. Webster and B. Pollara, *Comp. Biochem. Physiol.* 30, 509 (1969); E. Giblett, *Progr. Med. Genet.* 2, 34 (1962).
3. E. Baker and E. Morgan, *Biochemistry* 8, 1133 (1969); J. Fletcher, *Clin. Sci.* 37, 273 (1969); S. Kornfeld, *Biochim. Biophys. Acta* 194, 25 (1969).
4. C. Baker, *Comp. Biochem. Physiol.* 20, 949 (1967); J. Clark, D. Osuga, R. Feeney, *J. Biol. Chem.* 283, 3621 (1963); E. Ezekiel, L. Lai, E. Kaldor, *Comp. Biochem. Physiol.* 10, 69 (1963); E. Giblett, C. Hickman, O. Smithies, *Nature* 183, 1589 (1959).
5. D. Shreffler, *Proc. Nat. Acad. Sci. U.S.* 46, 1378 (1960); E. Morgan, *Aust. J. Exp. Biol. Med. Sci.* 47, 361 (1969); D. Gilten and A. Biasucci, *J. Clin. Invest.* 48, 1433 (1969); P. Toivanen, T. Rossi, T. Hirvonen, *Scand. J. Haematol.* 6, 113 (1969).
6. E. Baker and E. Morgan, *Quart. J. Exp. Physiol. Cog. Med. Sci.* 54, 173 (1969).
7. J. Mueller, O. Smithies, M. Irwin, *Genetics* 47, 1385 (1962); R. Brown and H. Sharp, *Anim. Blood Groups Biochem. Genet.* 1, 113 (1970).
8. A. Ogden, J. Morton, D. Gilmour, E. McDermid, *Nature* 195, 1026 (1962); A. Stratil, *Comp. Biochem. Physiol.* 24, 13 (1968); C. Baker, *Genetics* 58, 211 (1968).
9. F. Kristjansson, *Genetics* 48, 1059 (1963).
10. 0.075*M* phosphate and 0.075*M* NaCl (pH 7.2).
11. W. Levi, *The Pigeon* (Levi, Sumter, S.C., ed. 3, 1957), pp. 269–271; and personal observation.
12. I thank Dr. Ray Owen for valuable discussion, support, and criticism. Supported by AEC contract AT 04376706 FP and PHS grant GM 00086.

26 October 1970

Copyright © 1971 by the American Association for the Advancement of Science

ADDITIONAL DATA

In order to test further the identity of egg white and serum transferrin in the pigeon, antisera were prepared in rabbits to pigeon serum- and egg-white derived transferrins. The anti-egg white transferrin was prepared in a manner similar to that described for the antiserum to serum transferrin in the preceding publication. Egg white and serum transferrins were assayed by Ouchterlony double diffusion with both antisera. They gave lines of identity with both reagents (fig. 1). These sera, therefore, detect no difference between pigeon egg white and serum transferrins.

Ferguson, however, reported a difference in electrophoretic mobility between egg white and serum transferrin from feral pigeons (1). Samples of Pasadena pigeon egg white were sent to Dr. Ferguson. He found that Irish pigeons differ from U.S. pigeons in the characteristic of egg white transferrin mobility (personal communication). U.S. pigeon egg white transferrins have mobilities identical to serum transferrins, while in Irish pigeons the two transferrins differ. This confirms the assertion in the preceding paper that egg white and serum transferrins are identical in Pasadena pigeons.

REFERENCE

1. Ferguson, A.: Comp. Biochem. Physiol. 38B:477 (1971)

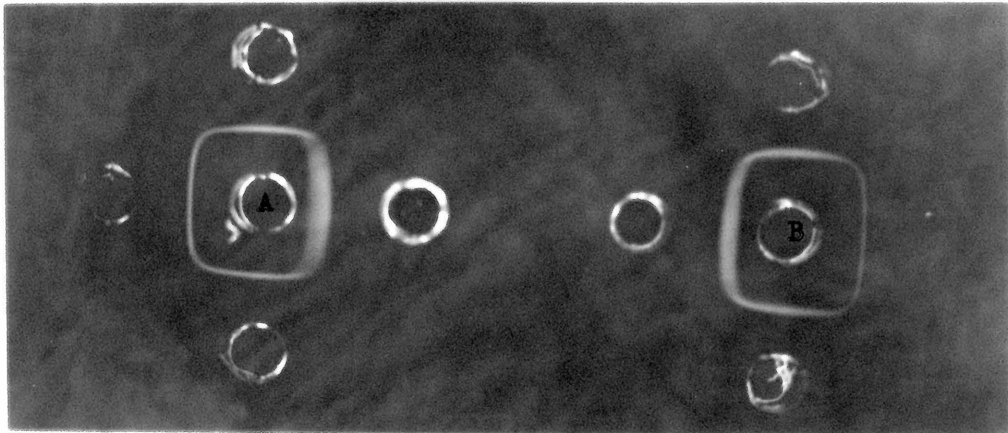

A
B

Figure Legend

Immunodiffusion test of the identity of serum and egg white transferrin. Top and bottom wells of each pattern contain purified serum transferrin; left and right wells purified egg white transferrin. The center well, A, contains anti-serum transferrin; the center well, B, anti-egg white transferrin.

CHAPTER 2

Maintenance of Transferrin Polymorphism in Pigeons

The publication contained in this chapter is from the Proceedings of the National Academy of Sciences and is reproduced here with the permission of the Academy.

Reprinted from
Proc. Nat. Acad. Sci. USA
Vol. 69, No. 2, pp. 326–329, February 1972

The Maintenance of Transferrin Polymorphism in Pigeons

(*Saccharomyces cerevisiae*/eggwhite)

JEFFREY A. FRELINGER

Division of Biology, California Institute of Technology, Pasadena, Calif. 91109

Communicated by Ray D. Owen, November 26, 1971

ABSTRACT **Transferrin, a nonheme iron-binding protein, is polymorphic in most vertebrate species that have been examined. In pigeons, it is controlled by an autosomal gene, with two known codominant alleles, Tf^A and Tf^B. The two alleles are found in nearly equal frequencies and the three genotypes are at Hardy–Weinberg equilibrium in all populations studied. This report shows that ovotransferrins from heterozygous females inhibit microbial growth, by use of yeast as an assay organism, better than ovotransferrins from either of the homozygous types, or those from a mixture of homozygous types. Heterozygous females hatch a larger percentage of their eggs than homozygous females. This difference is probably accounted for by the transferrin effect. The failure of the mixture of the homozygous types to act like the heterozygous type calls into question the currently accepted structure of transferrin as a monomeric protein. The greater fecundity of heterozygous females can account for the maintenance of transferrin polymorphism in pigeons.**

How genetic polymorphism is maintained is one of the major unanswered questions in genetics today. The belief that it depends upon a selective advantage of heterozygotes is widely held but not well supported by data. This paper presents evidence for maintenance of transferrin polymorphism in pigeons by the differential inhibition of microbial growth by different transferrin phenotypes.

Transferrin is a nonheme iron-binding protein found in the plasma of vertebrates and the eggs of birds. It is found to be polymorphic in most species examined. In each case appropriately studied, transferrin is found to be controlled by codominant alleles of an autosomal gene (1, 2). There have been many attempts to find mechanisms for the maintenance of this polymorphism. Ashton and his associates (3, 4) have reported evidence in some cattle for excess of heterozygotes, and postulated superior heterozygote fitness. This is not confirmed in other groups of cattle (5, 6), although lowered milk production of one homozygous class has been reported (6). In other domestic animals (pigs, mice), no effect of transferrin type on fertility has been found (7, 8). In *Microtus*, lowered fitness of both one homozygous and one heterozygous type has been suggested by field studies, but no selective mechanism has been proposed (9, 10). Recently, Morton and Gilmour have reported an association between maternal transferrin type and hatchability in chickens (11).

Mueller *et al.* (12) reported that there are two alleles, which act as Mendelian codominants, present in pigeon populations. These observations have been confirmed in population studies by others (13, 14) and in this laboratory by family studies. Starch gel electrophoresis used in our laboratory displays no difference in mobility between transferrins of different tissue sources in the same bird, so the term *transferrin* will be used to describe the molecule regardless of the tissue source. In all populations of pigeons that have been studied, frequencies of the two alleles have been close to 0.5 for each allele (13, 14). The fact that only two alleles exist, and that they exist in similar frequencies with the genotypes at Hardy–Weinberg equilibrium in such diverse places as Ireland, Wisconsin, Missouri, and California, suggests that powerful selective pressures may be operating to maintain this polymorphism.

The data reported here suggest that in pigeons, the polymorphism is maintained by fertility differences among females, due to greater resistance of the offspring of heterozygous females to embryonic and early posthatching infection. I have reported (15) that young squabs express, for a considerable period, not their own transferrin type but the maternal type. This interval corresponds closely to the period of immuno-incompetence, thus giving the squabs of heterozygous females an extended period of enhanced protection from microbial infection. Consideration of this kind of selection leads to a model that predicts maintenance of polymorphism in the absence of a discernible excess of heterozygotes in the population (Frelinger and Crow, manuscript in preparation).

MATERIALS AND METHODS

Pigeons. Two breeds of pigeon (*Columba livia*) were used in these studies, White Kings and Tumblers. The birds were maintained in pairs in mating cages or in flypens. All nests were checked for eggs three times a week. Eggwhites were collected from infertile eggs and stored frozen at −20°C until use. Eggwhites and sera were typed by starch gel electrophoresis as described (15).

Transferrin Purification. Pigeon transferrin was purified by dialyzing eggwhite against 5 mM sodium acetate (pH 5), centrifuging, and discarding the precipitate. The supernatant was chromatographed on carboxymethyl Sephadex C-25 in the same buffer. The breakthrough was pooled, 1 ml of 1 mM $FeCl_3$ in 0.5 M sodium carbonate was added, and the solution was dialyzed against 0.01 M Tris–0.06 M NaCl, and chromatographed on DEAE Sephadex A-25. This gave a product about 80% pure as judged by A_{280}/A_{470} ratios and by immuno-electrophoresis.

Yeast Growth. Wild-type diploid yeast, *Saccharomyces cerevisiae*, was grown on 1% casein hydrolysate, 1%

yeast nitrogen base (Difco), 2% dextrose, 2 mg of tryptophan per ml, 0.1 mg of biotin per ml, and 100 μg of streptomycin per ml. For the eggwhite experiments, this medium was made 10% eggwhite from a single typed eggwhite, filtered through a 0.3 μm Millipore filter, and 10 ml of this solution was inoculated with about 10^5 colony-forming units of yeast per ml. The yeast was grown in suspension at 30°C, in a shaking water bath. When purified transferrin was used, the protein was first dialyzed against 1000-fold excess of 0.01 M citric acid (pH 4.7) to remove the iron, followed by dialysis against 0.05 M morpholinoprane sulfonic acid buffer (pH 7.0). The medium was also made up in 0.05 M morpholinopropane sulfonic acid buffer and sterilized by filtration as before. The inoculum used for this experiment gave a final concentration of about 7 × 10^3 colony-forming units/ml. The final transferrin concentration was 2 mg/ml, approximately the same as a 10% eggwhite solution. Yeast growth was monitored by serial dilution and duplicate plating on 2% agar plates (1% yeast extract, 2% dextrose, and 2% peptone) at intervals during the growth period. Tests of statistical significance were "two-tailed" *t* tests.

RESULTS

Following the current convention for naming electrophoretic variants, the alleles described by Mueller as Tf^{L1}, Tf^{L2} (12) have been renamed as Tf^A and Tf^B, Tf^A controlling the faster-migrating transferrin. For the pigeons in this laboratory the allele frequencies are A = 0.52 and B = 0.48 (n = 97). These genotypes do not differ significantly from Hardy–Weinberg equilibrium in their distribution in the flock, or in either breed. The allele frequencies reported by Ferguson (14) in Northern Ireland were A = 0.592 and B = 0.408. Brown and Sharp (13) in Missouri reported A = 0.381 and B = 0.619. It appears that this polymorphism is both relatively stable and widespread.

Hatchability

The hatching records of the female birds in our colony were correlated with transferrin type of the females. The values for Tumblers are shown in Table 1. Kings have poorer performance in our flock, but show similar differences among female genotypes. A significantly greater portion of the eggs of AB females hatch than do the eggs of the two homozygous types.

Inhibition of yeast growth

Fig. 1 shows typical curves for the growth of yeast in medium containing individual eggwhites, all at the same eggwhite concentration. Variability of the AB inhibition was common among individual AB eggwhites, but in no case did AB inhibit growth less than the homozygous types. Table 2 shows

TABLE 1. *Hatchability of eggs*

Genotype of female	Number of eggs laid	Number of eggs hatched	% Hatched
AA	128	59	46
BB	144	75	52
AB	267	180	67

Chi square = 7.99; $P < 0.02$.

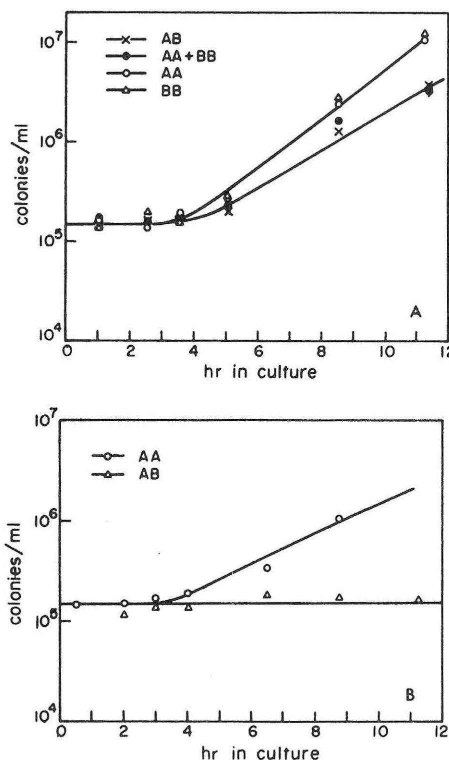


FIG. 1. Growth of yeast in medium containing eggwhite. (*A*) shows one of the experiments in which AB transferrin did not inhibit yeast growth greatly. (*B*) shows an experiment in which the AB transferrin totally inhibited the yeast growth, while the yeast grown in A tranferrin grew at its characteristic rate.

a composite of growth rates of these growth curve experiments. These rates are calculated by computation of the number of yeast doublings during the culture period divided by the elapsed time in hours minus the 2-hr lag phase. Addition of $FeCl_3$ at a concentration of 0.01 mg/ml abolished the differences among transferrin genotypes. The two homozygous types and, most interestingly, mixtures of the two types, give similar yeast growth rates. This finding suggests an interaction in the heterozygote, absent from the simple mixture of the homozygous eggwhites. However, mixtures of the two homozygous types are indistinguishable from the heterozygous type on starch gels.

Early experiments with purified transferrin in phosphate buffer failed to show the difference found with crude eggwhite. Apparently, this was due to iron contamination of the reagent-grade phosphate used. When the iron-free synthetic buffer morpholinopropane sulfonic acid was used, the difference again became apparent (Fig. 2). In these experiments it was necessary to pool 5 or 6 eggwhites of each type for the purification procedure. The yeast growth rates calculated in this experiment (Table 2) are almost identical to those calculated from the experiments with pooled raw eggwhite. This indicates that the effect observed in raw eggwhite is in fact due to transferrin.

DISCUSSION

These experiments show for the first time *in vitro* a differential inhibition of microbial growth by transferrin from different phenotypes. The bacteriostatic and fungistatic properties of transferrin are well documented, predating, in fact, the modern description of transferrin (16). Transferrin has been shown to inhibit a wide variety of iron-dependent microorganisms including *Saccharomcyes cerevisiae*, *Staphlococcus aureus*, *Candida albicans*, *Shigella dysenteria*, *Pasturella septica*, *Pseudomonas*, *Clostridium welchii*, *Lysteria moncytogens*, and *Salmonella typhimurium* (17). In chickens, embryonic death is frequently associated with microbial contamination of the egg (18). *Salmonella* can penetrate through the egg shell to contaminate the egg (19). Levi (20) has reported that while *Salmonella* infection may be asymptomatic in adults, it is a frequent cause of death in young squabs. He also cites *Candida albicans*, *Pasturella*, and *Mycobacterium tuberculosis* as common in pigeons. Thus, it appears that the opportunities for the infection of the egg and young squab are substantial, and the bacteriostatic and fungistatic properties of transferrin constitute, presumably, an important early line of defense. Because the transferrin both in the egg and the young bird is of maternal origin, this critical selective period is based on the maternal genotype rather than the embryo's own constitution. This leads to the survival of a greater proportion of the progeny of heterozygous mothers, but produces distributions of surviving genotypes indistinguishable from the Hardy–Weinberg equilibrium (Frelinger and Crow, manuscript in preparation).

Several workers have reported that transferrin is a monomeric protein (1, 2). The failure of the mixture of the two homozygous types to display the functional characteristic of the heterozygous type suggests the presence of an interaction product in the heterozygote. This is difficult to explain in terms of a monomeric protein, if the alleles represent a structural locus controlling the primary structure of the protein. The difference in the action of the heterozygous protein suggests the presence of dimeric protein, so that "hybrid" molecules can be produced in the heterozygote. In cattle, a second gene affecting the carbohydrate portion of the transferrin molecule has recently been reported by Spooner (21). If the difference between the allelic transferrins of pigeons is in their carbohydrate rather than in their primary protein structures, it then becomes possible to resolve the difference between heterozygous transferrin and the mixture of the two homozygous types. Experiments are currently in progress to determine if the inherited difference is in the amino acid or in the carbohydrate portions of the molecules.

TABLE 2. *Yeast growth in the presence of eggwhite and of purified transferrin of three types**

Transferrin type	Material	Growth rate	Number of egg-whites†
A (Tf^A/Tf^A)	Eggwhite	0.495 ± 0.0637	6
	Purified transferrin	0.34	5 (pool)
B (Tf^B/Tf^B)	Eggwhite	0.393 ± 0.0374	13
	Purified transferrin	0.30	5 (pool)
Mixture of A and B (Tf^A/Tf^A + Tf^B/Tf^B)	Eggwhite	0.425 ± 0.0509	4
AB (Tf^A/Tf^B)	Eggwhite	0.162 ± 0.067	10
	Purified transferrin	0.14	6 (pool)

* Gosset's t test for significance revealed no significant difference ($0.5 > P > 0.3$) between A and B. These data were therefore pooled and tested against the mixtures of A and B. This comparison also yielded no significant difference ($0.7 > P > 0.5$). Therefore, these data (A, B, mixture of A and B) were pooled and compared to AB. The difference is highly significant ($P < 0.0003$).

† For purified transferrin, number of eggwhites is the number of different eggwhites in the pool from which the transferrin was purified. In the case of eggwhites, it represents the number of individual egg whites that were separately tested. The results are given as the mean growth rate ± the standard error.

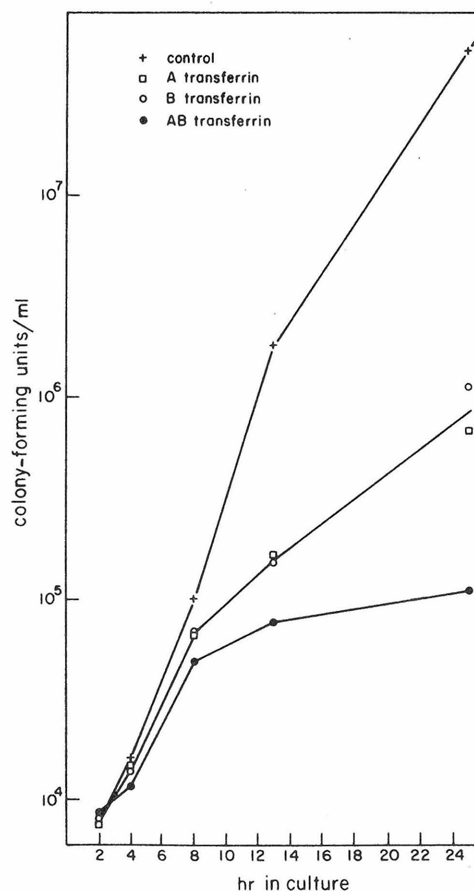


FIG. 2. Growth of yeast in purified transferrin. This shows clearly that while both A and B transferrins inhibit the growth as compared with the control, the AB type inhibits more than either homozygous type.

The results report for the first time a direct *in vitro* test of a proposed selective mechanism for the maintenance of a polymorphism. The data are consistent with the hypothesis that the polymorphism is maintained by selection against the offspring of homozygous females because of their greater susceptibility to infection both as embryos and as young birds. This interpretation is suggested by both the *in vitro* data on inhibition of yeast growth and the *in vivo* results represented by the hatchability data.

A preliminary report was presented at the Genetics Society of America meeting in August 1971. I thank Dr. Ray D. Owen for guidance and support during this work and Dr. H. K. Mitchell for suggesting morpholinopropane sulfonic acid buffer. This work was supported by Atomic Energy Commission contract AT(04-3)-767 and U.S. Public Health Service training grant GM 00086.

1. Feeney, R. & Komatsu, S. (1966) *Struct. Bonding (Berlin)* **1**, 146–207.
2. Baker, C. M. A., Crozier, G., Stratil, A. & Manwell, C. (1970) *Advan. Genet.* **15**, 147–174.
3. Ashton, G. C., Francis, J. & Ritson, J. B. (1966) *Aust. J. Biol. Sci.* **19**, 821–829.
4. Ashton, G. C. & Fallon, G. R. (1962) *J. Reprod. Fert.* **3**, 93–104.
5. Ashton, G. C. & Lampkin, G. H. (1965) *Genet. Res.* **6**, 209–215.
6. Fowle, K. E., Cline, J. H., Klosterman, E. W. & Parker, C. F. (1967) *J. Anim. Sci.* **26**, 1226–1231.
7. Nagai, J. & Kristjensson, K. (1970) *Can. J. Genet. Cytol.* **12**, 307–315.
8. Fesus, L. & Rasmusen, B. A. (1971) *Anim. Blood Groups, Biochem. Genet.* **2**, 57–58.
9. Tamerin, R. H. & Krebs, C. J. (1969) *Evolution* **23**, 183–211.
10. Gaines, M. S., Myers, J. H. & Krebs, C. J. (1971) *Evolution* **25**, 443–450.
11. Gilmour, D. G. & Morton, J. R. (1971) *Theor. Appl. Genet.* **41**, 57–66.
12. Mueller, J. O., Smithies, O. & Irwin, M. R. (1962) *Genetics* **47**, 1385–1392.
13. Brown, R. V. & Sharp, H. B., Jr. (1970) *Anim. Blood Groups, Biochem. Genet.* **1**, 113–115.
14. Ferguson, A. (1971) *Comp. Biochem. Physiol.* **38B**, 477–486.
15. Frelinger, J. A. (1971) *Science* **171**, 1260–1261.
16. Schade, A. L. & Caroline, L. (1944) *Science* **100**, 14–15.
17. Kochan, I., Golden, C. A. & Bukovic, J. A. (1968) *J. Bacteriol.* **100**, 64–70.
18. Quarles, C. L., Gentry, R. F. & Bressler, G. O. (1970) *Poultry Sci.*, **49**, 60–66.
19. Williams, J. E. & Whittemore, A. D. (1967) *Avian Dis.* **11**, Studying Microbial Penetration through the Outer Struc- 467–490.
20. Levi, W. (1957) in *The Pigeon* (Levi Publishing Co., Sumter, S.C.), 3rd ed., pp. 381–448.
21. Spooner, R. L. & Baxter, G. (1969) *Biochem. Genet.* **2**, 371–382.

CHAPTER 3

Transferrin Polymorphism and the Hardy-Weinberg Ratios

The publication in this chapter has been accepted for publication in the *American Naturalist*. It is included in the form in which it was accepted.

TRANSFERRIN POLYMORPHISM AND THE HARDY-WEINBERG RATIOS

The iron binding protein, transferrin, is polymorphic in almost all vertebrate species where it has been studied. In pigeons the two alleles, Tf^A and Tf^B, are present in approximately equal frequency in populations widely scattered throughout the world and the three genotypes are in Hardy-Weinberg (H.W.) proportions in each population (Ferguson 1971). A mechanism to account for the gene frequency stability has recently been proposed (Frelinger 1972). Eggs from heterozygous females inhibit microbial growth more strongly than those from either homozygote, and it is suggested that this probably accounts for the observed differences in egg-hatch from the three maternal genotypes. Because a larger fraction of their eggs hatch, heterozygous females are effectively more fertile than homozygotes, in that they contribute more offspring to the population, and this is sufficient to maintain the two alleles in balanced polymorphism.

It is known that strong selection can cause wide departures from H.W. ratios in a randomly mating population if the enumeration is made after selection, but not if this is done before. In this case selection acts through embryonic mortality so it might appear that enumeration of adults would show departures from H.W. ratios, especially in view of the strong selection that is implied by the large differences in egg hatch. But, the selection is based on the maternal genotype, not that of the embryo. We shall show that in this case the equilibrium population is in H.W. proportions, as observed. However, if the population has not reached gene frequency equilibrium there will be a slight excess of heterozygotes.

We assume that selection is acting solely by the mechanism just

described and designate the various parameters in a randomly mating population as follows:

Genotype	AA	AB	BB	Total
Adult frequency	P	2Q	R	1
Relative contribution of females	1-s	1	1-t	W
Relative contribution of males	1	1	1	1

$W = P(1-s) + 2Q + R(1-t) = 1 - sP - tR$

$p = P + Q$, the frequency of allele A

$q = Q + R$, the frequency of allele B

The frequencies of the three genotypes next generation will be

(1) $P' = (P + Q - sP)\ (P + Q)/W = (p^2 - spP)/W$

(2) $2Q' = [(P + Q - sP)\ (Q + R) + (Q + R - tR)\ (P + Q)]/W = (2pq - sqP - tpR)/W$

(3) $R' = (Q + R - tR)\ (Q + R)/W = (q^2 - tqR)/W.$

Adding (1) to half of (2) we obtain the A gene frequency next generation, which after some algebraic rearrangement is

(4) $p' = p + (tpR - sqP)/2W.$

There will be an equilibrium when $tpR = sqP$.

We use the quantity $H = Q^2/PR$ as a measure of the departure from H.W. proportions; with random mating proportions $H = 1$. We then write

$$(5) \quad H' = \frac{Q'^2}{P'R'} = \frac{(2pq - sqP - tpR)^2}{4(p^2 - spP)(q^2 - tqR)}$$

$$= \frac{p^2q^2 - pq(sqP + tpR) + (sqP + tpR)^2/4}{p^2q^2 - pq(sqP + tpR) + stpqPR}$$

Notice that the numerator and denominator are the same except for the last term. If we subtract one from the other we have

$$(6) \quad (sqP + tpR)^2/4 - stpqPR = (sqP - tpR)^2/4 \geq 0$$

Comparison of (6) and (4) shows that if the population is at gene frequency equilibrium it is in H.W. proportions, but until this stage is reached there will be an excess of heterozygotes.

Near equilibrium, $P \approx p^2$ and $R \approx q^2$, and (4) becomes

$$(7) \quad p' - p \approx (tpq^2 - sqp^2)/2W$$

$$= \frac{pq(s+t)}{2W}\left[\frac{t}{s+t} - p\right]$$

The equilibrium value of p (when $p' - p = 0$) is $t/(s+t)$, as in simple over-dominance for fitness. The equilibrium is locally stable, as can be seen from (7) because gene frequency change is always in the direction of the equilibrium value. This can be interpreted as a special case of fertility considerations (Bodmer, 1965).

The excess of heterozygotes in non-equilibrium populations implied by (6) is caused, not by differential mortality *per se*, but by the resulting unequal gene frequencies in the contribution of male and female parents. This corresponds to the standard principle that when the parental gene frequencies are unequal in the two sexes random mating leads to an excess of heterozygotes (Wright 1969, p. 4; Crow and Kimura 1970, p. 44). On the other hand, if the population sampled is actually made up of several randomly mating subpopulations with differing gene frequencies, there may well be an overall deficiency of heterozygotes.

The heterozygote excess shown by (6) is very slight in most cases. Figure 1 shows the situation graphically, where the population genotype frequencies are shown by a point in the triangle. The numerical values of s and t (.313 and .224) are based on the egg hatch data of Frelinger (1972). From any starting point the population moves in a single generation of random mating to a point to the left and below the H.W. parabola. Actually in this example the points are so close to the curve as to be indistinguishable in the graph. The population then follows the parabola to the point of gene frequency equilibrium. The final value is determined by the ratio of s to t, whereas the rate of approach is determined by their absolute values.

The fact that the departure from H.W. proportions is so slight during this process explains the regular finding of H.W. ratios, which then does not necessarily imply that the populations are in gene frequency equilibrium.

References

Bodmer, W. F. 1965. Differential Fertility in Population Genetic Models. Genetics 51: 411-424.

Crow, J. F., and M. Kimura. 1970. An Introduction to Population Genetics Theory. Harper.

Ferguson, A. 1971. Geographic and Species Variation in Transferrin and Ovotransferrin Polymorphism in Columbidae. Comp. Biochem. Physiol. 38B: 477-486.

Frelinger, J. A. 1972. The Maintenance of Transferrin Polymorphism in Pigeons. Proc. Nat. Acad. Sci. USA 69: 326-329.

Wright, S. 1969. Evolution and the Genetics of Populations. Vol. 2. The Theory of Gene Frequencies. University of Chicago Press.

Acknowledgments

We thank Dr. Ray D. Owen for advice and encouragement. JF was supported by USPHS Training Grant GM 0086, and AEC Contract No. AT 0437-67.

Jeffrey A. Frelinger

James F. Crow

Division of Biology

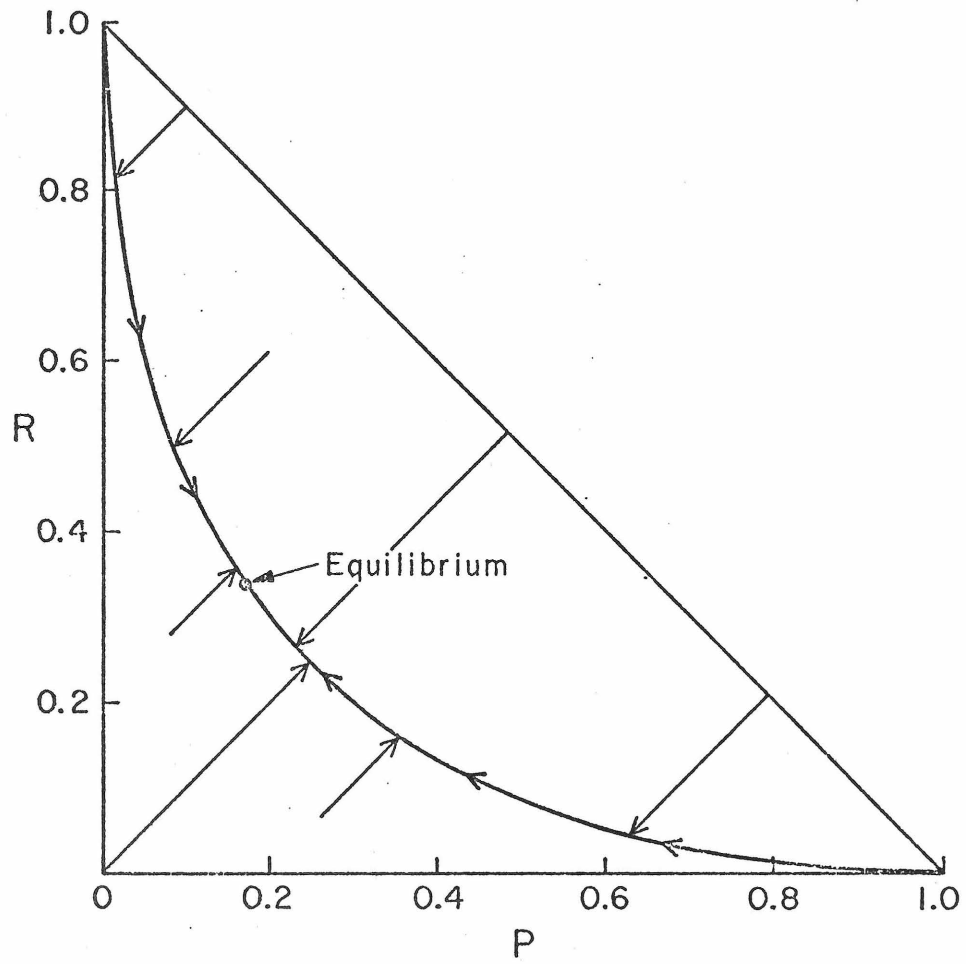
California Institute of Technology

Pasadena, California 91109

Laboratory of Genetics

University of Wisconsin

Madison, Wisconsin 53706



1.0
0.8
0.6
R
0.4
0.2
Equilibrium
0
0.2
0.4
0.6
0.8
1.0
P

Legend for Figure 1.

The approach to equilibrium for selection caused by maternally determined embryonic mortality. The population is represented by a point in the triangle. The proportion of AA is given by the horizontal distance from the R axis, the proportion of BB by the vertical distance from the P axis, and the proportion of AB by the horizontal or vertical distance to the diagonal. Populations in Hardy-Weinberg ratios lie on the parabola. The arrows represent the behavior of populations during the first generation of random mating. Afterward the population moves along the parabola to the equilibrium point. Although the departures are too small to show on the graph, the actual points are slightly below and to the left of the parabola, indicating a slight excess of heterozygotes until the equilibrium is reached.

CHAPTER 4

Chemical Basis of Transferrin Polymorphism in Pigeons

This chapter has been submitted for publication in Animal Blood Groups and Biochemical Genetics. It is included here in the form it was submitted.

CHEMICAL BASIS OF TRANSFERRIN POLYMORPHISM IN PIGEONS

Jeffrey A. Frelinger

Division of Biology, California Institute of Technology

Pasadena, California 91109

Transferrin is a non-heme iron-binding protein, polymorphic in most vertebrate species examined. Mueller _et al._ (1962) first reported electrophoretic variants of pigeon (Columba livia) transferrin. Frelinger (1972) has recently proposed that the gene frequency stability observed in pigeons is accounted for by superior inhibition of microbial growth by transferrins from heterozygotes. However, mixtures of homozygous type transferrins do not mimic transferrins from heterozygotes in this effect. For this reason we have investigated the chemical basis for the observed allelic variation. To our knowledge, the only species in which a chemical difference has been identified is in humans (Wang and Sutton, 1965; Wang _et al._, 1966; Jeppson and Sjöquist, 1966) although there have been other attempts (Roop and Putnam, 1967; Herschberger, 1970). We present data here suggesting a single amino acid difference between alleles in the two forms of pigeon transferrin.

MATERIALS AND METHODS

Eggs. Egg whites were collected, stored and typed as previously described (Frelinger, 1971; Frelinger, 1972). Five to ten whites of the same phenotype were pooled and purified by ion exchange and gel filtration chromatography (Frelinger, 1972). This preparation was > 99% pure as judged by SDS gel, cellulose acetate, starch gel, and immunoelectrophoresis.

Carbohydrate analysis. Purified transferrins were freed of carbohydrate contamination by gel filtration on Biogel P-100 in 0.06 M NaCl, 0.01 M Tris, pH 8.0. The peak was precipitated two times with 50% saturated ammonium sulfate, and two times with cold 50% ethanol. Carbohydrate was analyzed by gas chromatography as previously reported (Niedermeier, 1971). Sialic acid was determined by the method of Warren (Warren, 1959). The results of two independent preparations were averaged.

Reduction and carboxymethylation. Transferrin was reduced in 6 M guanidine HCl, 0.2 M 2-mercaptoethanol at 37°C for 4 hours and carboxymethylated by 10% excess of iodoacetamide, then extensively dialyzed against 0.2 M ammonium bicarbonate, and lyophilized.

Peptide mapping. TPCK trypsin (Worthington) was incubated with 6-8 mg reduced carboxymethylated protein suspended in 100 µl ammonium bicarbonate; 1 µl of trypsin (100 mg/ml in 0.001 M HCl) was added and the mixture was incubated for 1 hour at 37°C followed by the addition of 1 µl trypsin solution and a second hour of incubation. The mixture was lyophilized and suspended in 50 µl water, spotted and chromatographed in n-butanol/glacial acetic/water (540:160:800), followed by electrophoresis at pH 3.5 in pyridine-acetate buffer (Bennet, 1967). The maps were stained with ninhybrin-collidine stain. For elution the peptides were stained with 1% ninhydrin in ethanol. Peptides were located and eluted with water, or 50% (v/v) NH_4OH.

Amino acid analysis. Peptide eluates were lyophilized and hydrolyzed 18 hours in 6 N HCl at 110°C. The hydrolysates were analyzed on a Beckman 121C or Durrum D-500 amino acid analyzer. Total amino acid compositions were carried out similarly except that 24 and 48 hour hydrolysis times were used to enable the determination of serine and threonine content by extrapolation to zero time. Performic acid oxidized material (Hirs, 1967) was used to determine 1/2 cystines as cysteic acid. Tryptophan was analyzed by oxidation with N-bromosuccinimide (Spande and Witkop, 1967).

RESULTS AND DISCUSSION

No reliable differences were revealed in gross amino acid composition (Table 1) between the transferrins of the two homozygous types. Carbohydrate

analysis showed only glucosamine, mannose and galactose; no reliable differences were found between alleles. No sialic acid was detected. Repeated peptide mapping revealed only one consistent difference between A and B (Fig. 1), although other neutrally charged amino acid substitutions might be hidden. AB transferrins show both peptides. This peptide (T1), as well as six additional peptides, were eluted and their amino acid compositions determined (Table 2). T1 showed a striking difference in composition between the allelic types with a substitution of Asx in A for Ser in B. As this peptide also contained glucosamine I attempted to determine the carbohydrate composition of peptide T1 eluted from paper, but because of the large amount of free carbohydrate co-eluting, no reliable data resulted. Although the change from Ser- Asn is a single base change, and Ser - Asp is a two base change; I feel that the mobility of the intact protein and the peptide both suggest that the Asx in the peptide is an Asp. This indicates that the _Tf_ locus in pigeons codes for the transferrin structural gene rather than for a glycosyl transferase.

Earlier studies on the mechanism for the maintenance of transferrin polymorphism (Frelinger, 1972) suggests that the polymorphism is maintained by the superior inhibition of yeast

by transferrin produced in heterozygotes. One major unanswered question is how a simple amino acid substitution not directly involved as an iron binding ligand mediates a change in yeast inhibition. I suspect that this is due to a difference in iron binding between the alleles, and that this would explain the superior fungistatic effect of the heterozygous transferrin. Yeast serves,as a model for iron dependent pathogens, to explain the hatch differences among the eggs laid by females of different genotypes (Frelinger, 1972). This difference in fertility in its turn explains the maintenance of both alleles in stable gene frequency equilibrium(Frelinger and Crow, 1973). Another puzzle is the absence of the heterotic effect in yeast inhibition of simple mixtures of A and B protein. This argues for a qualitative difference in the molecules present in heterozygotes. A dimer structure for pigeon transferrin is indicated on biological grounds

SUMMARY

The structures of pigeon A and B ovotransferrins were compared by peptide mapping. A single identifiable difference was found between them. AB transferrin showed both peptides. Amino acid compositions of these peptides indicated an amino acid substitution Asx(in A) for Ser (in B).

ACKNOWLEDGEMENTS

This work was supported by USPH Training GM-0086 and AEC contact AT(04-3)-767. I thank Dr. Ray Owen for advice and support throughout this work. I thank Dr. Leroy Hood for his help and the generous use of his facilities for the amino acid analysis and peptide mapping, and Vincent Farnsworth and David Helphrey for technical assistance. I thank Drs. W. Niedermeier and M. Tomana for carbohydrate analysis.

LITERATURE CITED

Bennet, J. C., 1967 Paper chromatography and electrophoresis: special procedures for peptide maps. In: C.H.W. Hirs (Ed.), Methods in Enzymology, vol. 11, pp. 330-338. Academic Press, New York.

Frelinger, J. A., 1971 Maternally derived transferrin in pigeon squabs. Science 171: 1260-1261.

Frelinger, J. A., 1972 The maintenance of transferrin polymorphism in pigeons. Proc. Natl. Acad. Sci. U.S. 69: 326-329.

Frelinger, J. A. and J. F. Crow, 1973. Transferrin polymorphism and the Hardy-Weinberg ratios. Amer. Naturalist, in press.

Herschberger, W. K., 1970 Some physiochemical properties of transferrin in brook trout. Trans. Amer. Fish. Soc. 99: 207-218.

Hirs, C.H.W., 1967 Performic acid oxidation. In: C.H.W. Hirs (Ed.), Methods in Enzymology, vol. 11, pp. 197-198. Academic Press, New York.

Jeppson, J. O. and J. Sjöquist, 1966 Structural studies on genetic variants of human transferrin. In: H. Peeters (Ed.), Protides of the Biological Fluids, 14 Colloquium, Brugge, pp. 87-91. Elsevier, Amsterdam.

Mueller, J. O., O. Smithies and M. R. Irwin, 1962 Transferrin variation in Columbidae. Genetics 47: 1385-1392.

Niedermeier, W., 1971 Gas chromatography of neutral and amino sugars in glycoproteins. Anal. Biochem. 40: 465-475.

Roop, W. E. and F. W. Putnam, 1967 Properties of transferrin C and genetic variants. J. Biol. Chem. 242: 2507-2513.

Spande, T. F. and B. Witkop, 1967 Determination of tryptophan content of proteins with N-bromosuccinimide. In: C.H.W. Hirs (Ed.), Methods in Enzymology, vol. 11, pp. 498-505.

Wang, A. C. and H. E. Sutton, 1965 Human transferrins C and D_1: chemical difference in a peptide. Science 149: 435-437.

Wang, A. C., H. E. Sutton and A. Riggs, 1966 A chemical difference between human transferrins B_2 and C. Amer. J. Hum. Genet. 18: 454-458.

Warren, L., 1959 The thiobarbituric acid assay of sialic acid. J. Biol. Chem. 234: 1971-1975.

TABLE 1

Amino Acid Compositions

in Residues/80,000 molecular weight

Amino Acid	Tf^A/Tf^A	Tf^B/Tf^B	Tf^A/Tf^B
Lys	78	76	68
His	16	16	16
Arg	33	33	33
Asp (includes Asn)	77	77	80
Thr	38	36	38
Ser	54	55	49
Glu (includes Gln)	80	83	84
Pro	19	19	19
Gly	63	64	66
Ala	57	58	64
Val	50	53	53
Met	8	8	8
Ile	27	27	27
Leu	65	65	65
Tyr	25	26	23
Phe	27	27	27
1/2 Cys*	n.d.	34*	34*
Trp†	n.d.	n.d.	7

*Determined as cysteic acid after performic acid oxidation

†Determined by N-bromosuccinimide method

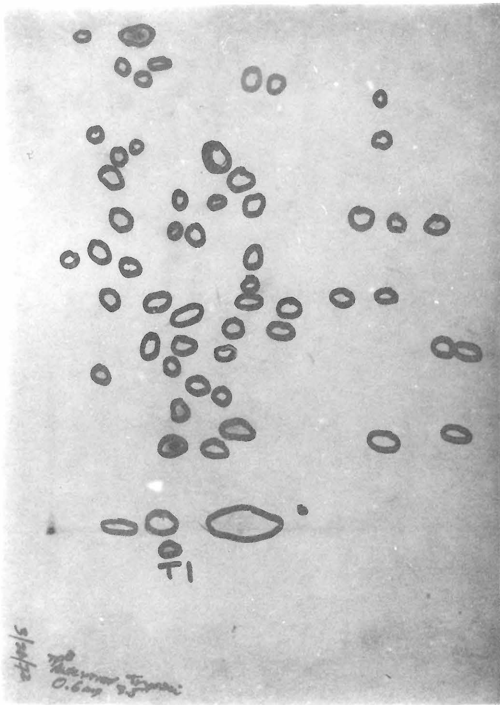
n.d. = not determined

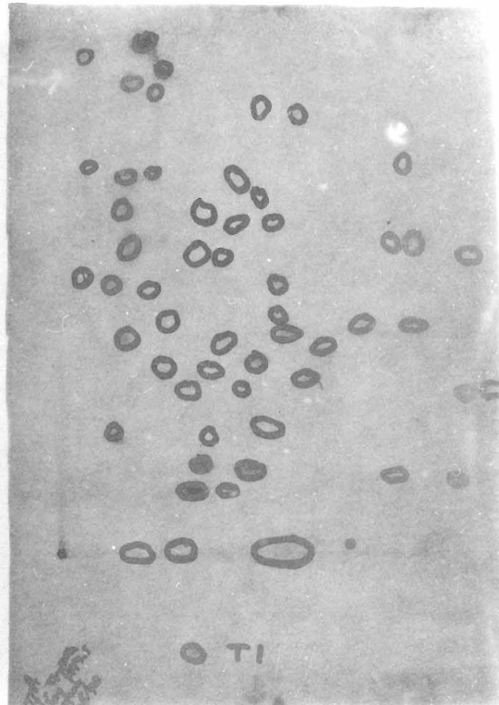
Table 2

Amino Acid Composition of Tryptic Peptides "T1"*

	A Peptide			B Peptide		
	n moles	Molar ratio	(nearest whole number)	n moles	Molar ratio	(nearest whole number)
Asp	7.1	2.5	2	2.6	.76	(1)
Thr	1.2	.42	(?)	1.5	.44	(?)
Ser	2.9	1.0	1	5.7	1.7	(2)
Glu	3.5	1.2	1	3.5	1.0	(1)
Gly	5.4	1.9	2	6.0	1.7	(2)
Ala	2.8	1.0	1	3.4	1.0	(1)
His	1.4	.52	(?)	2.3	.67	(?)
Lys	2.7	.96	1	3.2	.94	(1)

*Peptides prepared as described in text. Each figure is an average of three independent preparations, corrected for background, and calculated on the basis of Ala = 1.0. Trace amounts of other amino acids detected were 1/2 the level of Thr or less.





B
Ser

A
Asx

FIGURE LEGEND

Fig. 1. Representative tryptic peptide maps of reduced, carboxymethylated A and B allelic pigeon transferrins. Peptide T1 is indicated.

CHAPTER 5

The Covalent Structure of Transferrins

This chapter will be submitted for publication in *Biochemistry*. It is included here in substantially the form it will be submitted.

The Covalent Structure of Transferrins†

Jeffrey A. Frelinger‡, Jonathan S. Fuhrman and Leroy Hood*

Footnotes

†From the Division of Biology, California Institute of Technology, Pasadena, California 91109. Supported in part by NIH training grant GM-00086, AEC contract (04-3)-767, and a Career Development Award to L. H.

‡Present address: Department of Human Genetics, University of Michigan, Ann Arbor, Michigan.

*Author to whom correspondence should be addressed.

ABSTRACT: Pigeon transferrin was purified by sequencial ion exchange and gel filtration chromatography. The molecular weight and subunit structure was studied by sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis, gel filtration, amino sequence analysis, hydrazinolysis, succinylation, and cyanogen bromide cleavage. Chicken transferrin was examined in parallel. Pigeon transferrin was dissociated into 40,000 dalton fragments by SDS, urea, or succinylation. Chicken was unaffected. Sequence analysis indicated the possibility of multiple amino acid sequences in native pigeon transferrin. Contrary to the interpretation of a previous report sequence analysis of chicken cyanogen bromide fragments indicate that in spite of their size homogeneity each size class of cyanogen bromide fragments is heterogeneous. This indicates that the transferrin gene did not result from a recent fused tandem duplication.

Transferrin, a non-heme iron binding protein, is widely distributed in the serum and eggs of vertebrates. This molecule presents an interesting evolutionary problem. A majority of the molecular weight studies of transferrins suggest that they are single polypeptide chains of about 80,000 daltons (Greene and Feeney, 1968; Mann et al., 1970). A number of observations are consistent with the hypothesis that this polypeptide resulted from a fused tandem gene duplication. First transferrin has two identical carbohydrate moieties (Jamieson et al., 1971) and two equivalent, but independent iron binding sites (Aisen et al., 1967; Aasa et al., 1968), although this has recently been questioned (Luk, 1971). Second, in a number of species fewer tryptic peptides have been found (50-70%) than expected from the amino acid composition of a polypeptide of 80,000 daltons (Williams, 1962; Jeppson, 1967). Finally the cyanogen bromide fragments from chicken transferrin add up to a molecular weight of 40,000 daltons--half the expected size (Phillips and Azari, 1971).

Two other types of observations have, however, raised the possibility that at least certain transferrins may have a dimeric structure. First, at least three reports have suggested that the transferrins of man, cattle and hagfish are comprised of two 40,000 dalton subunits (Jeppson, 1967; Emfremov et al., 1971; Palmour and Sutton, 1971). Second, Frelinger (1972) has suggested that the mechanism for the maintenance of transferrin polymorphism in pigeons is mediated by the following mechanism: transferrin synthesized in birds heterozygous at the Tf locus inhibit yeast (and presumably other microbial) growth more

effectively than either of the homozygous types. The failure of simple mixtures of transferrins from the homozygous pigeons to mimic the heterozygote effect suggests that two or more subunits may be present in pigeon transferrin.

In this paper we report that pigeon transferrin can, under certain conditions, be broken down into 40,000 dalton subunits. In these experiments chicken transferrin, which appears under all conditions to be a single polypeptide of 80,000 daltons, is used as a control protein.

Materials and Methods

Transferrin Purification. Pigeons were maintained and eggs collected as previously described (Frelinger, 1971). Egg whites were separated from yolks and stored frozen until used. Six to 10 egg whites of identical phenotype were pooled and dialyzed against 0.005 M sodium acetate adjusted with acetic acid to pH 5.0. The precipitated mucin was removed by centrifugation at 27,000g. The supernatant containing the transferrin was chromatographed on Sephadex C-25 (∿200 ml Column volume) equilibrated with the same buffer. The breakthrough peak was collected, 1.0 ml of 0.0002 M ferric chloride in 0.5 M sodium citrate 0 .5 M sodium bicarbonate was added, and the resultant mixture was dialyzed against 0.06 M NaCl .01 M Tris pH 8.0. The material was concentrated by pressure using an Amicon XM-50 membrane. This material was chromatographed on A-25 Sephadex (200 ml Column volume). The breakthrough peak was pooled and concentrated. If this material appeared homogeneous by immunoelectrophoresis, it was then chromatographed on

Sephadex G-100 (1 cm diameter x 100 cm long column), and eluted as a single symmetrical peak. If not, it was rechromatographed on A-25 once again before chromatography on G-100. Final purity was assessed by SDS gels (Shapiro *et al.*, 1967; Weber and Osborn, 1969), disc gels and immunoelectrophoresis. Chicken ovotransferrin was purchased from Sigma Chemical and was chromatographed on G-100.

Amino Acid Analysis. Proteins were hydrolyzed in evacuated tubes (<20 in Torr) in 6 N HCl for 110° for 20-24 hr and examined on either a Beckman Model 121C Amino Acid Analyzer or a Durrum D-500 Amino Acid Analyzer.

Automatic Sequence Analysis. Protein and peptide fragments were analyzed on a Beckman Model 890 Sequencer using a program similar to that proposed by Edman and Begg (1967). An internal standard [pheny-thiohydantoin (PTH) norleucine] was added to each sample so that approximate corrections could be made for losses incurred during the various handling and analytic procedures. PTH amino acids were identified by gas and thin layer chromatography. The PTH amino acids were then hydrolyzed with 65% HI *in vacuo* for 20 hr at 110° and the resulting amino acids identified by amino acid analysis. The details of sequenator operation and analytic procedures are given in Hood *et al.* (1972).

Cyanogen Bromide Cleavage. Cyanogen bromide cleavage and the separations of the resulting peptide fragments were carried out as described by Phillips and Azari (1971). The cyanogen bromide fragments from pigeon transferrin appeared somewhat smaller than their chicken

counterparts and were, accordingly, chromatographed on Biogel P-100 rather than P-150.

SDS Gels. SDS gels were run as described by Shapiro et al. (1967). The following proteins were used as molecular weight standards: chymotrypsin, bovine serum albumin, human IgG, and ovalbumin.

Hydrazinolysis. Hydrazinolysis was carried out as described by Braun and Schroeder (1966). The resulting free carboxyterminal amino acids were identified by amino acid analysis.

Complete Reduction and Alkylation. Reduction was carried out at a protein concentration of 2 mg/ml in 6 M guanidine HCl-0.1 M Tris-0.2 M mercaptoethanol at pH 7.5 for 5 hr at 37°. Carboxymethylation was achieved by adding a 2-fold excess of iodoacemide over mercaptoethanol present at room temperature while keeping pH 7.5 by addition of Tris.

Succinylation. Succinylation was carried out at an initial protein concentration of 2 mg/ml in 4 M urea, pH 9.5. Succinic anhydride (2 mg/ml protein) was added slowly over 15 min while stirring on a pH autotitrator adjusted to pH 9.5 at room temperature. The reaction was continued for 10 min more and extensively dialyzed against .1% SDS, .01 M Na phosphate, 10% glycerol, pH 7.

Results

Molecular Weights and Chemical Modifications. Upon complete alkylation of half cystine residues, pigeon and chicken transferrin give an apparent molecular weight of 80,000 $\pm$ 2000 on SDS acrylamide gels. However, when pigeon transferrin is incubated in .1% SDS-phosphate

(.01 M, pH 7.0) for several days at room temperature, or when heated to 100° in this same buffer for 1 hr, approximately one-half of the 80,000 dalton material is converted to 40,000 daltons (see Figure 1). This reduction in molecular weight could be made quantitative by several procedures: incubation of the protein in 10 M urea (.01 M phosphate pH 7 overnight at room temperature); succinylation or addition of succinic acid in 4 M urea; and prolonged incubation in .1% SDS .01 M-phosphate buffer, either at room temperature or -20°. These treatments did not affect the apparent molecular weight of the chicken transferrin (Figure 2). To detect proteases which might be present in low amounts in the pigeon transferrin, we incubated ^{14}C-carboxymethylated chicken transferrin with 1% SDS phosphate buffer in the presence of unlabeled pigeon transferrin which had been totally converted to the 40,000 dalton form. No change in the apparent molecular weight of the chicken transferrin was noted.

In an attempt to further characterize the 40,000 dalton fragment of pigeon transferrin, SDS was removed by the method of Lenard (1971), and chromatography was carried out on Sephadex G-100 in .01 M Tris and .06 M NaCl at pH 8. This material eluted as a single symmetrical peak at position corresponding to approximately 80,000 daltons. When this material was analyzed again on SDS acrylamide gels, most of the polypeptide was 80,000 daltons.

Sequence Analysis of Intact Transferrins. The yields of PTH derivatives at the first step for the transferrins were low, ranging from 20-27%. In addition, the background noise was unusually high. In

Tables I and II are given the amino acids present (nanomoles) at steps 1 through 5 for chicken and pigeon transferrin respectively. The major residue alternatives for each position can be obtained by looking for residues which increase at one residue position and decrease at the next. Chicken transferrin appears to have a single major sequence whereas the pigeon transferrin appears to be heterogeneous at one or more positions (boxed residues in Tables I and II). The major sequences are as follows:

	1	2	3	4	5
Chicken transferrin	Ala-	Pro-	Pro-	Lys-	Ala-
Pigeon transferrin	Ala-	Thr-	Glu- Pro Thr	Lys-	Ala-

The reason for the unusually low yields of lysine at position 4 are uncertain--perhaps, in part, a result of specific losses during the acid hydrolysis steps.

These data, while unsatisfactory from a technical point of view, do suggest that the chicken and pigeon transferrins differ by two or more residues in five at the amino terminus. These data suggest that there may be two or more subunits in pigeon transferrin although better data would be necessary to verify this supposition.

Hydrazinolysis of Intact Transferrins. Hydrazinolysis of pigeon and chicken transferrins yielded 1 mole of serine per 80,000 daltons of protein after correction for hydrolytic losses according to Braun and Schroeder (1967) (Table III).

Analysis of Cyanogen Bromide Fragments. The elution patterns of the cyanogen bromide fragments of chicken transferrin were similar to

those previously reported (Phillips and Azari, 1971). Four fragments were seen by SDS acrylamide electrophoresis of approximate molecular weights 20,000, 10,000, 6000 and 3000. The two smaller fragments coeluted from the Biogel P-150 column. These fragments sum up to about 40,000 daltons. The amino acid compositions of the cyanogen bromide fragments from the chicken transferrin were also in general agreement with those of Phillips and Azari, 1971. Four cyanogen bromide fragments were also seen with SDS gels from pigeon transferrin, although each appeared on SDS gels to be slightly smaller than its chicken counterpart. The pigeon cyanogen bromide fragments I and II are similar in amino acid composition to those of the chicken. Fragment III differs particularly in proline and glutamic acid content (Table IV).

<u>Sequence Analysis of Chicken Transferrin Cyanogen Bromide Fragments.</u> Quantitative data for the residues at positions 1-5 are given in Tables V, VI and VII for cyanogen bromide fragments I, II, and III of chicken transferrin. Fragment I clearly contains the N-terminal sequence Ala-Pro-Pro-Lys-Ala- and also has one or more additional sequences, namely Asp-Ala-$\genfrac{}{}{0pt}{}{\text{Thr}}{\text{Glu}}$-Leu-Tyr- (compare Tables I and V). Cyanogen bromide fragment II also appears to have at least two major sequences, namely--Ala $\genfrac{}{}{0pt}{}{\text{Leu}}{\text{Glu}}$ $\genfrac{}{}{0pt}{}{\text{Pro}}{\text{Ile}}$ $\genfrac{}{}{0pt}{}{\text{Tyr}}{\text{Glu}}$ Asp--and probably one or more minor sequences. Fragment III appears to be rather heterogeneous, particularly at the N-terminal 2-3 positions.

<u>Absorption Spectroscopy</u>. The visible absorption spectra of pigeon ovotransferrin is shown in Figure 3 (dashed line). After reassociation of SDS treated material, and addition of iron to the preparation, the

absorption peak of the iron transferrin complex was markedly shifted to shorter wavelengths (solid line).

Discussion

We have confirmed in general the data of Phillips and Azari concerning the size distribution of cyanogen bromide fragments of chicken transferrin. However we find no evidence of sequence homogeneity within each size class of chick cyanogen bromide fragments. If they were closely related, only a single sequence would be expected, within each fragment. This argues that if the transferrin gene was derived from a fused tandem duplication, the event was in the distant past. This differs significantly from the interpretation of Phillips and Azari (1971). The data on chicken transferrin continue to unequivocally support the notion that it is made up of a single polypeptide chain. No evidence of dissociation to 40,000 daltons was found under any of the conditions employed in this study.

The study of pigeon transferrin has resulted in some contradictory data. Quantitative carboxyterminal amino acid analysis detects only a single amino acid, serine per 80,000 molecular weight. The possibility remains that a second carboxyterminal amino acid, if it were basic or amide amino acid, would be undetected by these techniques. The dissociation by SDS, or urea, argues that the protein is a dimer.

There are possible objections to this interpretation. It is known that SDS may allow the action of proteolytic factors (Pringle, 1970). Two lines of evidence argue against this. Pringle (1970) advocates

heating the SDS protein solution in boiling water immediately on dissolving. He finds that this removes the proteolytic activity for yeast hexokinase subunits. When we heat the protein-SDS solution in a boiling water bath, we find it speeds the dissociation. Preboiling the SDS solution for 10 min has no effect. Further, the SDS solution does not have this effect on chicken transferrin, or any of the other protein standards which we used. The second line is that the protein dissociates in urea, overnight, or by succinylation, giving independent evidence of its dissociation. Phosphoglucomutase, a protein earlier regarded as 62,000 daltons has recently been shown to dissociate in the presence of SDS to 31,000 dalton subunits while remaining 62,000 daltons in 6 M guanidine HCl (Duckworth and Sanwal, 1972). This is similar to the reactions observed with pigeon transferrin.

Further evidence in favor of the dimer argument is the reversible dissociation of native pigeon transferrin in SDS. When SDS is removed from this 40,000 M.W. preparation it reassociates to 80,000 M.W. When iron-citrate is added, the protein produces a chromogenic shift, which is however markedly different from the normal chromophore. We are not sure whether this represents a true alteration of the iron binding site, or is merely an artifact of small amounts of SDS still present in the preparation. Chicken transferrin when treated with SDS and then freed of SDS by similar methods also exhibits an absorption shift to shorter wavelengths, but only of 10-15 nm, much less than the 100 nm in the pigeon.

When pigeon transferrin is dissolved in urea and run on SDS gels it has an apparent molecular weight of 80,000. Only after standing overnight does its molecular weight decrease. The hydrazinolysis data suggest only a single COOH terminal. The presence of a multiple, related amino terminal sequence suggests several possibilities for the structure of pigeon transferrin. First is that the dissociation in SDS and urea is an artifact, and the protein is a single continuous polypeptide chain. This might be caused by hydrolysis of a labile peptide or unkown bond. Another possibility is the presence in pigeon transferrrin preparations of an enzyme specific for pigeon transferrin, capable of cleaving only a site near the middle of the protein. Another is that the molecule is a dimer composed of similar subunits, whose second C terminal amino acid is a basic or amide amino acid, and therefore undetected. The chemical data on this point are not decisive, although the results of the sequence experiments may be compatible with a dimeric model. Arguments from homology favor a single poplypeptide model for the structure of pigeon transferrin.

The biological data previously reported indicate that the activity of heterozygous protein is qualitatively different from that found in simple mixtures of homozygous proteins. Further study showed that the difference between the allelic types was a single amino acid substitution. Carbohydrate analysis of the two types was inconclusive, but suggested no difference between the allelic types (Frelinger, in preparation). The biological data can be explained if the pigeon transferrin is a dimer, which would allow the production of hybrid moleculesin the heterozygote. This molecule would possess one subunit of each type

and therefore be qualitatively different from simple mixtures. This apparent dissociation in SDS and urea, as well as the sequence data are consistent with this interpretation while the hydrazinolysis data and homology from chicken argue against the dimeric interpretation. Further work will be needed to answer this question with certainty.

Acknowledgments

We thank Dr. Ray Owen for advice and support. We thank David Helphrey and Vincent Farnsworth for technical assistance. We thank Dr. Paul Hargrave for his critical review of this manuscript.

References

Aasa, R., Malmström, B. G., Saltman, P., and Vänngård, T. (1963), Biochim. Biophys. Acta 75, 203.

Aisen, P., Aasa, R., Malmström, B. G., and Vänngård, T. (1967), J. Biol. Chem. 242, 2484.

Braun, V., and Schroeder, W. A. (1967), Arch. Biochem. Biophys. 118, 241.

Duckworth, H. W., and Sanwal, B. D. (1972), Biochemistry 11, 3182.

Edman, P., and Begg, G. (1967), Eur. J. Biochem. 1, 80.

Emfremov, G. D., Smith, L. L., Barton, B. P., and Huisman, T. H. J. (1971), Anim. Blood Groups, Biochem. Genet. 2, 159.

Frelinger, J. A. (1971), Science 171, 1261.

Frelinger, J. A. (1972), Proc. Nat. Acad. Sci. U.S. 69, 326.

Greene, F. C., and Feeney, R. E. (1968), Biochemistry 7, 1366.

Hood, L., McKean, D., Farnsworth, V., and Potter, M. (1972), Biochemistry. In press.

Jamieson, G. A., Jett, M., and DeBernado, S. L. (1971), J. Biol. Chem. 246, 3686.

Jeppson, J. O. (1967), Acta Chem. Scand. 21, 1686.

Lenard, J. (1971), Biochem. Biophys. Res. Comm. 45, 662.

Luk, C. K. (1971), Biochemistry 10, 2838.

Mann, K. G., Fish, W. A., Cox, A. C., and Tanford, C. (1970), Biochemistry 9, 1348.

Palmour, R. M., and Sutton, H. E. (1971), Biochemistry 10, 4026.

Phillips, J. L., and Azari, P. (1971), Biochemistry 10, 1160.

Pringle, J. R. (1970), Biochem. Biophys. Res. Comm. 39, 46.

Shapiro, A. L., Viñuela, E., and Maizel, J. V., Jr. (1967), Biochem. Biophys. Res. Comm. 28, 815.

Weber, K., and Osborn, M. (1969), J. Biol. Chem. 244, 4406.

Williams, J. (1962), Biochem. J. 83, 355.

TABLE I: Sequence of Native Chicken Transferrin.

Amino Acid	Step: 1	2	3	4	5
HIS	1.5	1.2	.7	.74	.95
ARG	1.3	1.4	0	1.4	0
TYR	1.1	.98	0	1.4	1.6
PHE	1.2	1.7	1.3	1.6	2.0
LYS	0	1.1	1.3	10.6	3.2
PRO	0	10.3	7.2	3.0	0
GLY	.87	.49	1.3	1.2	2.3
ALA	25.5	5.4	6.5	6.3	16.6
THR	.65	.49	0	.7	1.4
VAL	.35	.8	1.6	2.2	2.9
ILE	.83	0	.53	0	.97
LEU	1.1	1.5	1.8	2.3	3.5
ASP	.81	.60	.77	2.3	2.4
GLU	.76	1.1	1.0	1.8	1.95

TABLE II: Sequence of Native Pigeon Transferrin.

Amino Acid	Step: 1	2	3	4	5
HIS	2.97	1.87	1.74	1.86	2.86
ARG	0.00	3.75	2.20	3.31	3.56
TYR	6.72	6.09	6.25	6.61	10.06
PHE	6.98	6.14	5.43	6.87	10.37
LYS	3.68	5.60	3.88	9.86	12.50
PRO	18.42	19.68	19.05	14.15	25.00
GLY	13.27	15.38	10.60	15.20	17.15
ALA	51.60	41.30	32.93	37.40	65.81
THR	6.47	18.90	15.78	12.62	22.77
VAL	12.78	10.30	11.41	12.65	19.90
ILE	7.20	7.38	4.86	5.30	7.33
LEU	11.40	14.64	12.39	14.39	21.41
ASP	11.38	9.25	6.68	8.69	11.58
GLU	14.86	15.34	19.00	18.83	24.76

TABLE III: Hydrazinolysis of Transferrins.

Protein	nm Expected[a]	nm Found[b]	%	Amino Acid
Pigeon transferrin	38	37	99	SER
Chicken transferrin	37	33	88	SER
Cytochrome c	188	164	87	GLU
BSA	280	192	72	ALA

[a]Per molecular weight. 80,000 assumed for transferrins.

[b]Corrected for destruction during hydrolysis.

TABLE IV: Amino Acid Composition of Cyanogen Bromide Fragments.

Amino Acid	CNBr I	CNBr II	CNBr III	Sum of CNBr Fragments	Native Protein
	Residues/Molecule				
Chicken					
LYS	17	6	6	29	58
HIS	5.3	1.3	.84	7	14
ARG	8.5	3.1	4.4	16	33
ASP	19	9	9	37	74
THR	10.9	4.9	3.5	19	39
SER	10.7	5.9	4.0	21	49
GLU	17.2	8.3	8.2	34	71
PRO	8.6	3.8	3.8	16	29
GLY	13.3	6.7	4.9	25	50
ALA	14.6	6.2	4.3	25	53
VAL	10.9	5.1	6.9	23	46
ILE	7.2	2.4	2.5	12	25
LEU	11.5	6.0	6.1	23	52
TYR	6.0	3.3	1.5	11	23
PHE	5.7	3.7	2.2	11	25
Pigeon					
LYS	17	6	6	29	67
HIS	2.9	.69	.5	5	14
ARG	9.1	2.0	2.5	14	30
ASP	19	9	9	37	70
THR	9.2	2.1	6	17	34
SER	9.6	4.6	3.6	19	49
GLU	19.8	11.0	16.8	46	73
PRO	7.0	3.6	0	10	17
GLY	16.1	3.4	8.5	30	58
ALA	14.0	4.7	8.6	26	54
VAL	11.2	6.5	10.7	29	47
ILE	4.8	3.8	2.7	13	22
LEU	15.2	8.9	4.5	29	59
TYR	3.9	2.7	1.9	9	23
PHE	7.1	3.7	0.99	12	25

TABLE V: Sequence of Chicken Cyanogen Bromide Fragment I.

Amino Acid	Step: 1	2	3	4	5
HIS	0.44	0.73	0.47	2.64	2.25
ARG	1.07	1.44	1.73	0.0	0.0
TYR	2.76	3.71	2.32	8.58	12.83
PHE	3.45	3.29	2.52	9.27	7.47
LYS	2.84	2.91	1.74	15.12	9.28
PRO	4.44	9.27	6.31	16.20	12.48
GLY	8.75	6.75	4.23	19.62	14.14
ALA	29.72	25.71	13.05	48.88	47.74
THR	2.67	4.54	7.05	12.44	12.36
VAL	4.39	4.57	4.10	13.96	12.57
ILE	3.35	3.58	3.15	8.36	6.91
LEU	7.91	8.29	7.72	36.95	18.05
ASP	15.99	5.16	3.46	15.76	15.78
GLU	6.71	8.53	12.25	24.94	16.04

TABLE VI: Sequence of Chicken Cyanogen Bromide Fragment II.

Amino Acid	Step: 1	2	3	4	5
HIS	0.91	0.00	0.94	1.77	0.88
ARG	4.77	0.00	0.00	0.00	5.06
TYR	1.14	7.69	2.82	29.32	6.53
PHE	21.94	9.23	5.79	3.32	2.65
LYS	8.62	4.13	3.59	6.50	3.95
PRO	2.83	25.74	28.49	17.14	11.74
GLY	54.01	15.38	12.75	10.02	5.89
ALA	124.33	21.07	51.19	35.92	36.08
THR	3.60	3.33	4.97	4.00	5.09
VAL	5.20	4.02	5.35	4.16	5.43
ILE	4.56	4.18	22.61	7.86	4.67
LEU	6.70	68.99	20.86	13.49	13.44
ASP	20.60	12.77	10.26	8.52	84.04
GLU	8.43	67.80	23.74	41.14	14.19

TABLE VII: Sequence of Chicken Cyanogen Bromide Fragment III.

Amino Acid	Step: 1	2	3	4	5
HIS	1.20	0.00	0.00	0.00	0.00
ARG	9.24	5.10	0.00	0.00	0.00
TYR	2.34	15.77	1.68	2.66	5.45
PHE	7.72	4.74	16.09	4.18	0.00
LYS	40.38	48.92	6.23	2.88	5.10
PRO	56.09	65.59	0.00	6.61	0.00
GLY	17.12	22.12	8.31	6.61	11.68
ALA	48.91	17.45	27.41	17.74	27.76
THR	7.66	2.28	0.00	4.92	0.00
VAL	8.48	9.59	9.65	4.80	17.87
ILE	10.22	9.47	4.16	4.24	0.00
LEU	53.80	12.65	23.93	23.39	90.38
ASP	72.55	40.17	59.65	8.93	13.57
GLU	21.74	14.39	22.38	104.18	11.08

Figure Legends

FIGURE 1: Scans of SDS acrylamide gel patterns of pigeon transferrin. Top pattern native protein, bottom after heating solution for 1 hr at 100°.

FIGURE 2: Effect of 10 M urea on transferrins. Transferrins labeled with ^{14}C iodoacemide were electrophoresed on SDS acrylamide gels, frozen, sectioned, dispersed in NCS (Amerschen Searle) and counted. Top pattern is chicken transferrin, bottom from pigeon transferrin. Similar patterns were obtained after succinylation.

FIGURE 3: Visible absorption spectra of pigeon transferrin.

FIGURE 4: Elution pattern of CNBr cleaved pigeon transferrin. Hydrolysate was chromatographed on Biogel P-100 in 1 M proprionic acid.

Figure 1

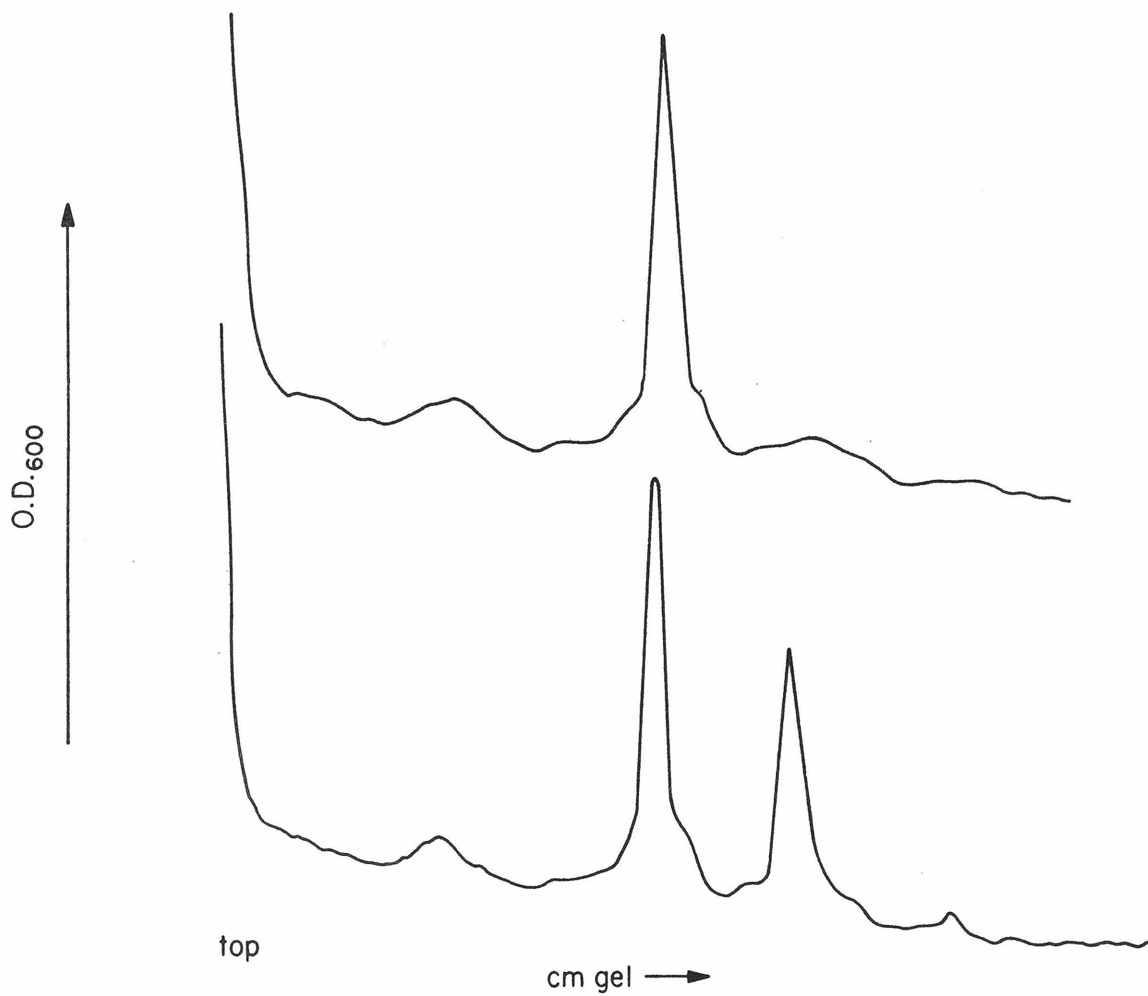


Figure 2

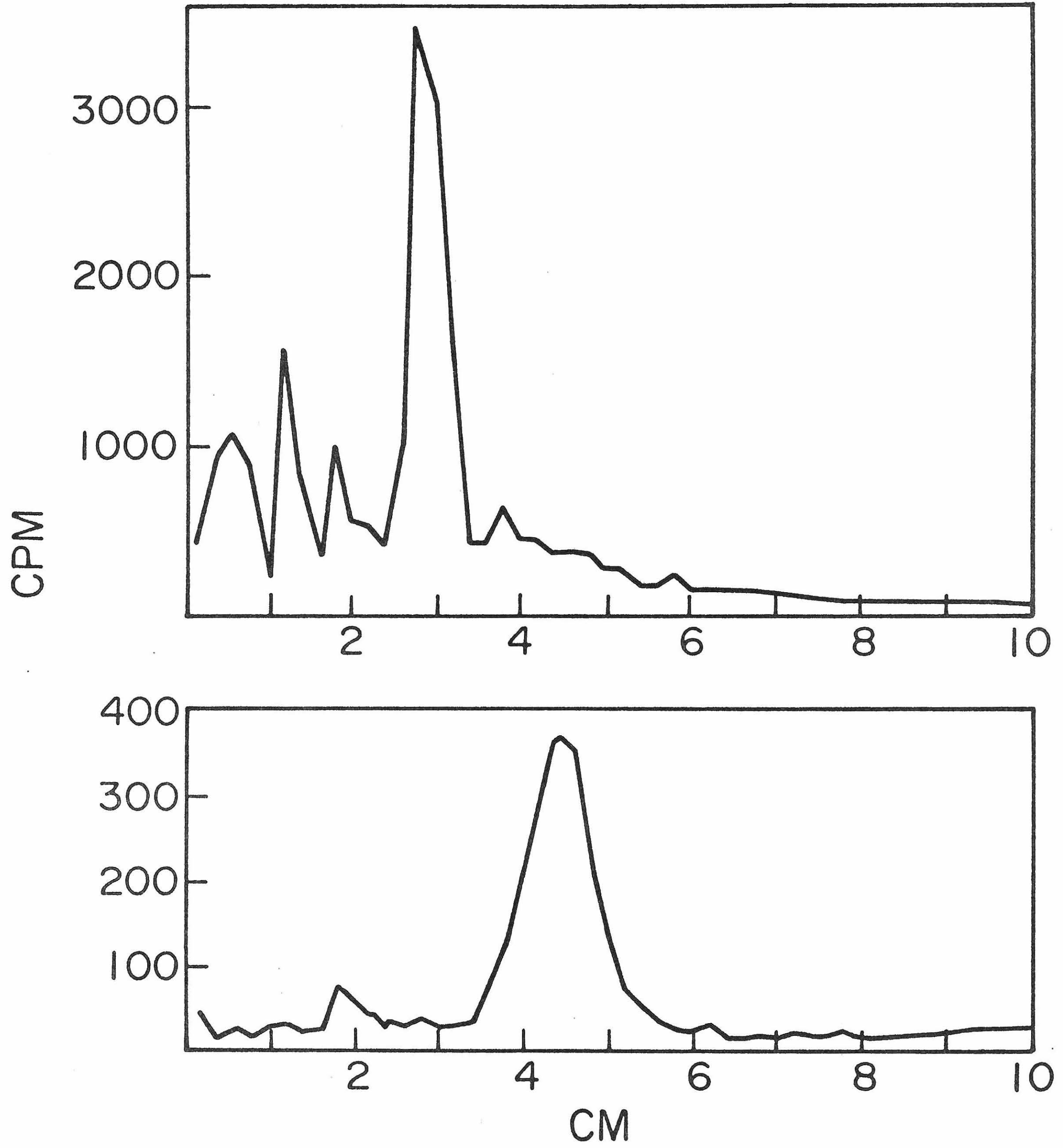


Figure 3

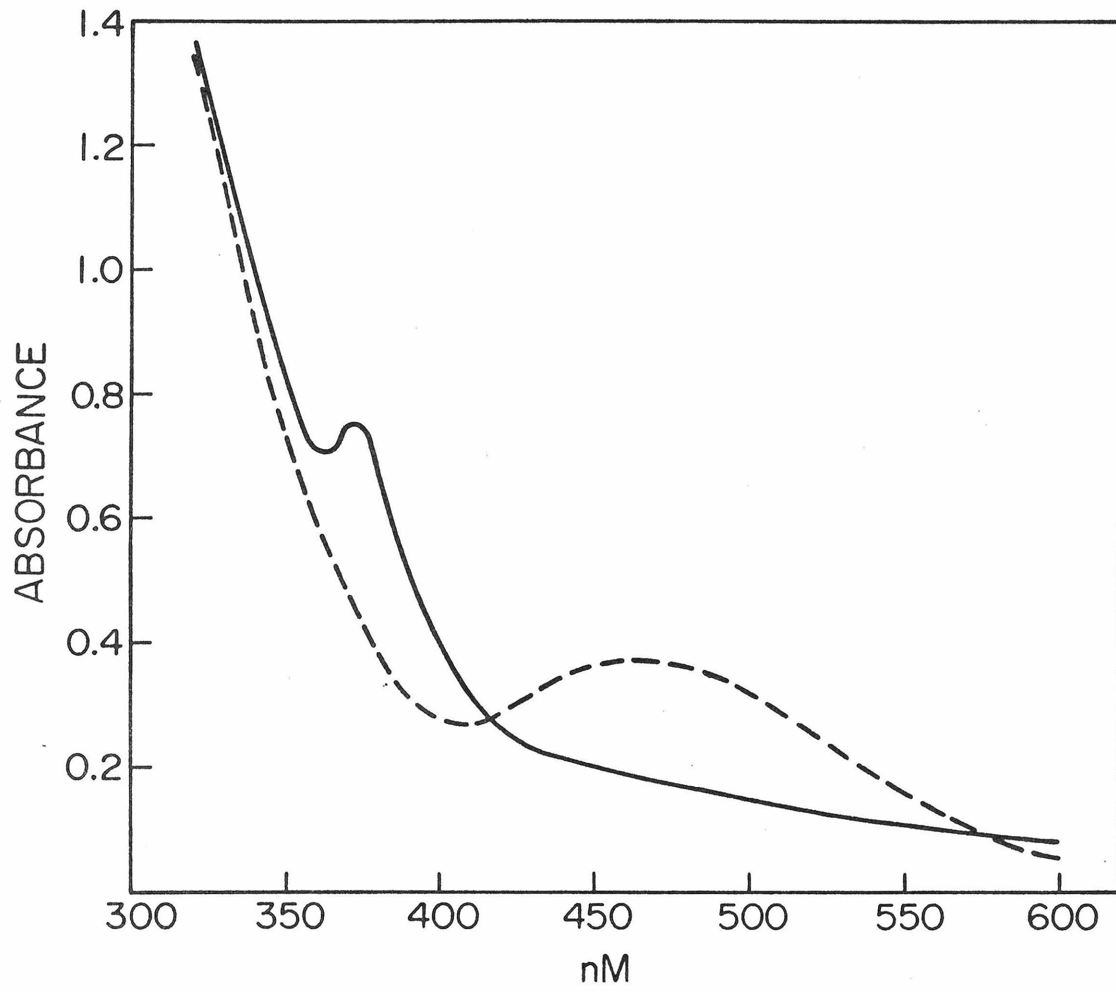
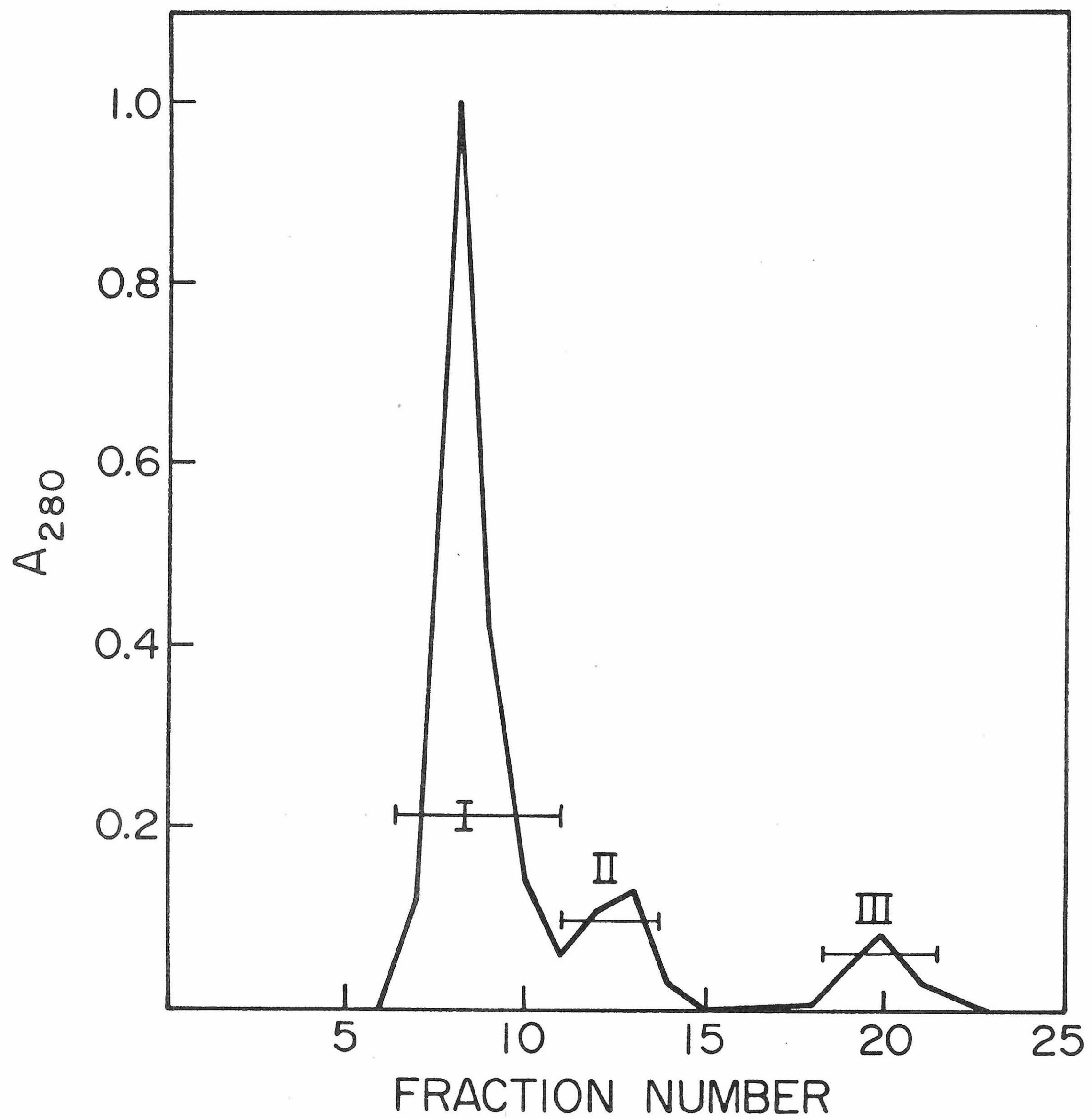


Figure 4



CHAPTER 6

Summary and Recapitulation

This thesis is addressed to the problem of the maintenance of polymorphisms in populations--the raw material of evolution. Currently two explanations are being considered for stable polymorphisms. The first is that polymorphisms are maintained by selective factors operating on individuals. The best known example of this explanation is sickle cell hemoglobin. The lethality of one allele when homozygous (SS) is balanced by increased resistance to malaria in heterozygotes (SA) as compared with the other homozygous class (AA). There are more of an excess of the number of heterozygotes in a population, than would be predicted by Hardy-Weinberg proportions. Other types of selection, such as differential fertility with no associated mortality, can result in a stable polymorphisms without heterozygote excesses. The second explanation for stable polymorphism has recently been put forward by Kimura. He suggested that many polymorphisms could be maintained by recurring neutral mutations, and random genetic drift. He believed that many of the electrophoretic variants detected are of neutral value. This idea is particularly attractive because of the scarcity of any data supporting heterosis and of defined rational and relevant selective mechanisms.

In this thesis, I have presented evidence for a selective mechanism for maintaining transferrin polymorphism in a balanced fashion by a simple heterotic effect. Pigeon transferrins are a diallelic series which differ in electrophoretic mobility. They represent a system of alleles which could be maintained in a population

as a balanced polymorphism. The egg hatch data presented here, for a population under stress, suggest large differences in selective coefficients among transferrin phenotypes. The proposed selective mechanism operates during the period from shell deposition through embryogenesis and hatching and for about one week afterward. The immune protective system is not competent over much of this interval. The developing bird reflects its maternal phenotype rather than its own genotype, so that the selection acts on the maternal rather than the offspring's own genetic makeup. This presents a rational selective scheme on the level of the intact organism. The heterozygous female produces a transferrin better at inhibiting iron-requiring pathogens, as modeled in the studies with yeast. This decreases embryonic and early post-hatching mortality in her offspring, regardless of their own genotypes, and is reflected in hatch differences of the eggs laid by the different classes of females. On the population level, heterozygous females obviously produce equal numbers of A and B gametes. Therefore, it makes intuitive sense that the gene frequency equilibrium is set by the differences between the selection coefficients for homozygous classes. These are the same relationships that the selection coefficients would have if selection were operating on progeny in the conventional manner rather than maternal genotype as in this case. However, in this case, unlike the conventional situation, the population is in Hardy-Weinberg equilibrium and fails to show any excess of heterozygotes at gene frequency equilibrium.

The biological data contained a compelling contradiction, which led to a consideration of the molecular basis of transferrin structure. On starch gels, transferrins from heterozygotes look like simple mixtures of the two homozygous types. But, in the yeast inhibition assay, mixtures of the homozygous types of protein do not mimic the action of the heterozygous transferrins; and the postulated selective advantage of heterozygous females requires that the heterozygous types be superior to either homozygote. This immediately suggests two possibilities. One is that the Tf gene in the pigeon is a glycosyltransferase. This would allow two different carbohydrate groups to be attached to a single transferrin molecule in heterozygotes, while simple mixtures of homozygotous proteins would contain no such molecules. This seems unlikely in view of the finding, reported in Chapter 4 of this thesis, that there is a simple amino acid difference between A and B. The single amino acid difference suggests that the allelic difference is in the transferrin structural gene rather than in a gene for a glycosyltransferase.

The second possibility is that transferrin is a dimer. A dimeric structure would permit the formation of hybrid molecules in a heterozygote, which would not be produced by simply mixing the two molecules.

The chemical data bearing on the structure of pigeon transferrins are still equivocal. The protein apparently dissociates

with prolonged incubation with SDS, urea, or succinylation into a 40,000 molecular "monomer". This is reversible, with at least partial restoration of iron binding activity. However, the amino and carboxyl terminal data suggest only one residue per 80,000 M.W. The sequence data, therefore, suggest a single predominant sequence. Thus, the question of whether there are one or two polypeptides chains must remain open.

This thesis poses some further interesting questions. How does a single amino acid substitution affect the iron binding site to produce a change in yeast inhibition? How is the protein in heterozygotes really different from mixtures of the homozygotes? What is the precise nature of the interaction between the protein and the microbe? What is the nature of the transport process of transferrin into the embryo? What is the evolutionary history of the transferrin gene?