Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 879–883, February 1982
Genetics

Evolution of alkaline phosphatases in primates

(gene duplication/gene expression/placenta/lung/inhibition)

DAVID J. GOLDSTEIN, CAPRICE ROGERS, AND HARRY HARRIS

Department of Human Genetics, University of Pennsylvania School of Medicine, 195 Med Labs/G3, Philadelphia, Pennsylvania 19104

Contributed by Harry Harris, October 13, 1981

ABSTRACT **Alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] in placenta, intestine, liver, kidney, bone, and lung from a variety of primate species has been characterized by quantitative inhibition, thermostability, and immunological studies. Characteristic human placental-type alkaline phosphatase occurs in placentas of great apes (chimpanzee and orangutan) but not in placentas of other primates, including gibbon. It is also present in trace amounts in human lung but not in lung or other tissues of various Old and New World monkeys. However, a distinctive alkaline phosphatase resembling it occurs in substantial amounts in lungs from Old World monkeys but not New World monkeys. It appears that duplication of alkaline phosphatase genes and mutations of genetic elements controlling their tissue expression have occurred relatively recently in mammalian evolution.**

At least three gene loci code for the various forms of human alkaline phosphatase [ALPase; orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1]: one for the polymorphic placental forms, at least one for the intestinal forms (adult and fetal), and at least one for the liver, bone, and kidney forms (1–4). In a recent investigation of ALPases in various laboratory and domestic animals, evidence for only two such loci, one coding for liver/bone/kidney ALPase and the other for intestinal ALPase, was obtained (5). The ALPase expressed in placenta in these species was quite different from human placental ALPase and corresponded to the liver/bone/kidney ALPase in each species and also in man. This result led to the hypothesis that the expression of human placental-type ALPase appeared relatively late in mammalian evolution, subsequent to the divergence of the evolutionary lineage leading to man from the lineages leading to these mammalian species. This hypothesis prompted the present study, in which we have examined ALPases from a variety of tissues in a diverse series of primates. Our findings indicate that the appearance of human placental-type ALPase in placenta was indeed a late event in mammalian evolution. It apparently occurred subsequent to the divergence of the lineages leading to man and the great apes (chimpanzee and orangutan) from the lineages leading to lower primates, including gibbon, which is classified with man and the great apes in the Hominoideae, but in a separate taxonomic category. Others have reported the occurrence of a heat-stable human placental-type ALPase in placentas of chimpanzee and orangutan (6) and also somewhat unexpectedly in baboon lung (7). We have found that the heat-stable ALPase in baboon lung also occurs, in substantial amounts, in lungs from other Old World monkeys. It resembles human and chimpanzee placental ALPase immunologically but differs quite markedly in certain of its inhibition characteristics, indicating significant structural differences at least at the inhibitor binding sites. We have not found this ALPase in detectable amounts in lung from several New World monkeys. Nor is an ALPase with the same inhibition characteristics present in human lung. We have found, however, that human lung contains trace amounts of an ALPase with the characteristic features of human placental ALPase. It represents on average about 3.5% of total lung ALPase activity. These findings, taken as a whole, pose intriguing questions concerning gene duplication and the regulation of gene expression in multilocus enzyme systems.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Tissue samples from a variety of primates were obtained at autopsy, except for placentas, which were collected at delivery. The specimens were stored at −20°C until extracted. The various analytical methods used have been described: tissue extraction (1), ALPase assay (1), thermostability studies (1, 8), inhibition studies (5, 8), and Ouchterlony double-diffusion studies and staining of precipitin lines (10). Rabbit anti-human placental ALPase was a gift from Clive A. Slaughter. In the studies to determine the characteristics of heat-stable ALPase in various tissues, the tissue extract was preheated at 65°C for 1 hr and then centrifuged prior to analysis of the supernatant. When heat-stable residual ALPase activity was very low, the supernatant obtained after heating and centrifugation was concentrated 10-fold or more by using Minicon concentrators (Amicon).

RESULTS

Placental ALPase

Thermostability Studies. Human placental ALPase is remarkably thermostable. It may be heated at 65°C for an hour or more without loss of activity, in contrast to liver, bone, kidney, and intestinal ALPases, which are inactivated under these conditions (11, 12). We found that the ALPase in chimpanzee placenta is as thermostable as human placental ALPase. Thermostability studies on the ALPase in two orangutan placentas suggested that two major components are present (Fig. 1). One, accounting for 25–35% of the total ALPase activity, is thermostable like human and chimpanzee placental ALPase. The other is thermolabile like liver/bone/kidney ALPase. The ALPase in placentas from other primates (gibbon, rhesus, pigtailed macaque, squirrel monkey, and owl monkey) are all very thermolabile and indistinguishable in this respect from human liver/bone/kidney ALPase and the ALPase from liver, bone, and kidney from a variety of primate species.

Immunological Studies. Antiserum raised in rabbits against purified human placental ALPase crossreacts with intestinal ALPase (13, 14). However, when intestinal ALPase is tested against placental ALPase in Ouchterlony double-diffusion plates the crossreaction is seen to be one of only partial identity. The crossreacting antibodies can be adsorbed out with intestinal

Abbreviations: ALPase, alkaline phosphatase; Har, L-homoarginine; Leva, levamisole.

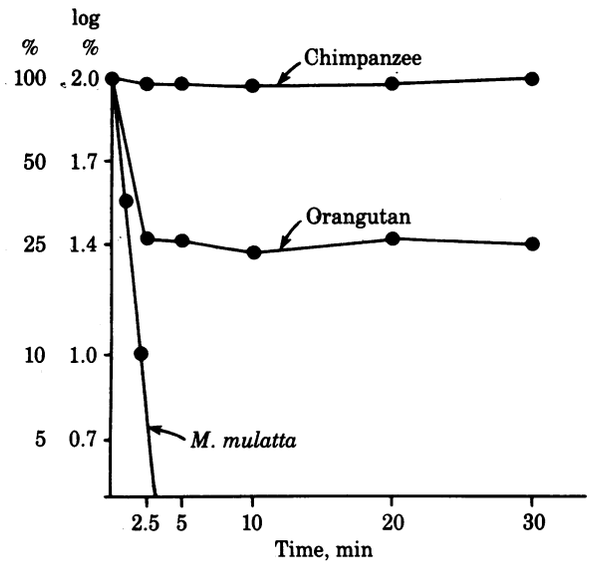

FIG. 1. Thermostability studies at 65°C on ALPase from chimpanzee, orangutan, and rhesus monkey (*Macaca mulatta*) placentas. Each point represents the log % of the original activity remaining after heating for the particular time.

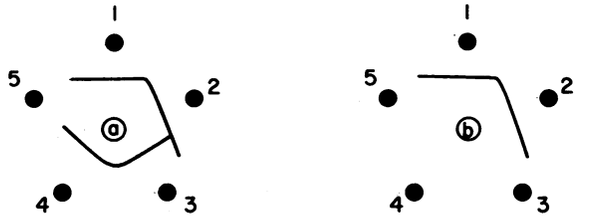

FIG. 2. Composite diagram of Ouchterlony double-diffusion plates showing precipitin lines obtained with antiserum raised in rabbit against purified human placental ALPase and with the same antiserum after it had been absorbed with human intestinal ALPase. *Center wells*: a, unabsorbed antiserum; b, absorbed antiserum. *Outer wells*: 1, human placental ALPase; 2, chimpanzee *or* heat-stable orangutan placental ALPase; 3, human intestinal ALPase; 4, intestinal ALPase from various primates (baboon, chimpanzee, rhesus monkey, etc.; see Table 1, § footnote); 5, placental ALPases from various primates other than chimpanzee and orangutan (see Table 1) and also liver ALPase from man and various primates.

ALPase, leaving antiserum reacting only with placental ALPase. These antisera do not crossreact with liver, bone, or kidney ALPases, so one may infer that intestinal ALPase is more closely related immunologically to placental ALPase than is liver/bone/kidney ALPase. We tested such absorbed and unadsorbed antisera against various primate ALPases by the Ouchterlony double-diffusion technique (Fig. 2). The results can be summarized as follows: (*i*) With both antisera, chimpanzee placental ALPase and the heat-stable orangutan placental ALPase gave reactions of apparent complete immunological identity with human placental ALPase and with one another. (*ii*) Other primate placental ALPases, including gibbon ALPase, showed no crossreaction with either antiserum, nor was there any crossreaction with primate liver ALPase. (*iii*) With the unabsorbed antisera the various primate intestinal ALPases, including those from chimpanzee and orangutan, showed crossreactions of partial immunological identity with human, chimpanzee, and heat-stable orangutan placental ALPase, but apparently complete immunological identity with one another. They did not crossreact with the absorbed antiserum.

Inhibition Studies. It is possible to discriminate sharply between human placental, intestinal, and liver/bone/kidney ALPase by inhibition studies using various amino acids, peptides, and other low molecular weight substances as inhibitors (1, 9, 15–21). We have used L-phenylalanine (Phe), L-homoarginine (Har), L-phenylalanylglycylglycine (Phe-Gly-Gly), levamisole (Leva), and L-leucine (Leu) as inhibitors and have determined for each case the concentration of inhibitor required to give 50% inhibition, [I_{50}], under standardized conditions (8). Table 1 summarizes the findings on placental, intestinal, and liver/bone/kidney ALPases from man and various primates. In man, the liver, bone, and kidney ALPases give the same [I_{50}]s

Table 1. Inhibition of ALPase in various tissues of various primate species

			[I_{50}], mM									
			Phe		Har		Phe-Gly-Gly		Leva		Leu	
Tissue	Species	*n*	*m*	SD	*m*	SD	*m*	SD	*m*	SD	*m*	SD
Placenta	Man	15	1.1	0.2	>50.0	—	0.10	0.02	1.7	0.8	5.7	0.3
	Chimpanzee	8	1.5	0.4	>50.0	—	0.11	0.03	1.3	0.5	7.8	0.9
	Orangutan*	2	2.6	0.6	>50.0	—	0.20	0.01	1.9	0.2	13.9	0.6
	Gibbon	1	30.3	—	3.1	—	29.3	—	0.03	—	9.7	—
	Rhesus	6	29.2	6.6	3.1	0.2	25.7	8.7	0.03	0.01	13.6	1.2
	Pig-tailed macaque	5	24.8	1.8	3.2	0.1	21.1	6.9	0.03	0.01	12.6	3.1
	Owl monkey	1	25.8	—	3.7	—	29.3	—	0.03	—	14.6	—
	Squirrel monkey	1	21.6	—	4.2	—	34.3	—	0.06	—	13.5	—
Liver/bone/kidney	Man	15	31.0	6.4	2.7	0.3	30.6	5.5	0.03	0.007	13.1	0.9
	20 primate spp.†	47‡	28.6	5.7	2.7	0.9	34.8	7.4	0.04	0.009	13.4	2.6
Intestine	Man	15	0.8	0.1	40.0	4.4	3.7	0.4	6.8	1.8	3.6	0.5
	10 primate spp.§	10	2.0	0.91	>50.0	—	8.4	4.7	3.1	1.5	7.5	3.4

Results are given with mean *m* and SD.

* Heat-stable fraction (residual ALPase after heating tissue extract at 65°C for 1 hr).

† Chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla*), gibbon (*Hylobates* sp.), orangutan (*Pongo pygmaeus*), mangabey (*Cercocebus* sp.), African green monkey (*Cercopithecus aethiops*), Celebese black ape (*Cynopethicus niger*), Java monkey (*Macaca fascicularis*), rhesus monkey (*M. mulatta*), pig-tailed macaque (*M. nemestrina*), bonnet monkey (*M. radiata*), barbary ape (*M. sylvanus*), mandrill (*Mandrillus sphinx*), baboon (*Papio* sp.), owl monkey (*Aotus trivirgatus*), marmoset (*Callithrix* sp.), capuchin (*Cebus capucinus*), tamarin (*Saguinus* sp.), squirrel monkey (*Saimiri sciureus*), and woolly monkey (*Lagothrix lagothricha*).

‡ Liver from 20 species, kidney from 16 species, and bone from 11 species.

§ Baboon, chimpanzee, rhesus, pig-tailed macaque, bonnet monkey, owl monkey, squirrel monkey, tamarin, capuchin, and marmoset.

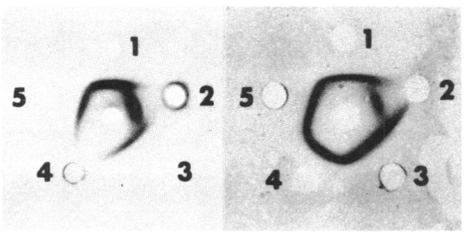

FIG. 3. Photograph of Ouchterlony double-diffusion plates, with precipitin bands stained for ALPase activity. *Center wells*: rabbit antiserum to purified human placental ALPase. *Outer wells*: (*Left*) 1, human placental ALPase; 2, human intestinal ALPase; 3, rhesus monkey lung ALPase; 4, rhesus monkey placental ALPase; 5, pig-tailed macaque lung ALPase. (*Right*) 1, human placental ALPase; 2, human intestinal ALPase; 3, heat-stable fraction of human lung ALPase; 4, Java monkey lung ALPase; 5, heat-stable fraction of human lung ALPase.

with each inhibitor. These ALPases are more sensitive by more than one order of magnitude to inhibition with Har and Leva than is human placental or intestinal ALPase. In contrast, human placental and intestinal ALPase are about 30 times more sensitive to inhibition with Phe than is liver/bone/kidney ALPase. Inhibition with Phe-Gly-Gly discriminates all three ALPase classes. Placental ALPase is about 30 times more sensitive to this inhibitor than is intestinal ALPase and is about 300 times more sensitive than is liver/bone/kidney ALPase. Chimpanzee placental ALPase and the heat-stable ALPase in orangutan placenta show essentially the same inhibition profile as human placental ALPase. In contrast, the ALPase in other primate placentas (gibbon, rhesus monkey, pig-tailed macaque, owl monkey, and squirrel monkey) show inhibition profiles like those of human and primate liver/bone/kidney ALPase. Intestinal ALPases from various primates, including chimpanzee and orangutan, differ from liver/bone/kidney ALPase by their relative insensitivity to Har and Leva and from human, chimpanzee, and orangutan heat-stable placental ALPases by their relative insensitivity to Phe-Gly-Gly.

Thus the placental ALPase in placenta from these various primates, with the exception of chimpanzee and orangutan, is quite different from human placental ALPase. Instead it closely resembles the liver/bone/kidney ALPase in the same and other mammalian species and also human liver/bone/kidney ALPase.

Lung ALPase

Following the report of a heat-stable ALPase in baboon lung similar immunologically to human placental ALPase (7), we studied lung ALPase from a series of different primates. After heating at 65°C for 1 hr, substantial quantities of residual ALPase activity were found in lung specimens from four Old World monkeys, baboon (59% of original activity), Java monkey (74%), rhesus monkey (76%), and pig-tailed macaque (50%), but no appreciable residual activity (<0.5%) was found in lung specimens from four New World monkeys (capuchin, marmoset, owl monkey, and tamarin). The heat-stable ALPase in the lungs of the Old World monkeys crossreacted with anti-human placental ALPase serum both before and after its absorption with human intestinal ALPase, giving a precipitin line of apparent identity when tested in Ouchterlony double-diffusion plates against human placental ALPase (Fig. 3). Thus this Old World monkey lung ALPase is immunologically more closely related to human placental ALPase than is human intestinal ALPase or the intestinal ALPase of these and other primates. However, its inhibition profile is markedly different from that of human placental-type ALPase (Table 2), being much less sensitive to inhibition by Phe, Phe-Gly-Gly, and Leu. It differs from primate and human liver/bone/kidney ALPase by its relative insensitivity to Har and Leva and from the intestinal ALPase in the same species by its relative insensitivity to Phe and Leu and by increased sensitivity to Phe-Gly-Gly. Thus it appears to have a unique inhibition profile with these five inhibitors. The differences from the three standard ALPase types imply significant differences in the structures of binding sites on the enzyme molecules for the various inhibitors. The $[I_{50}]$ values for the five inhibitors did not differ significantly among the four species in which the lung ALPase was found. The thermolabile ALPase in the lungs of the four New World monkeys gave an inhibition profile indistinguishable from that of liver/bone/kidney ALPase.

In view of these results, we examined a series of 21 autopsy samples of human lung from different individuals who died traumatically and showed no malignancy. In all cases most of the activity was thermolabile, and inhibition studies indicated that it was mainly liver/bone/kidney ALPase. But we consistently found a low level of residual ALPase activity after heating the specimens at 65°C for 1 hr. It amounted, on average, to about 3.5% of the original ALPase activity. This heat-stable ALPase fraction crossreacted with anti-human placental ALPase both before and after absorption with intestinal ALPase, and when tested against human placental ALPase in Ouchterlony double-diffusion plates it showed a line of continuity with no spurring (Fig. 3). Its $[I_{50}]$ values with the five inhibitors did not differ significantly from the corresponding values for human placental ALPase. Electrophoretic studies (to be reported elsewhere) of the series of heated lung extracts showed polymorphic differences similar to those observed in the well-established placental ALPase electrophoretic polymorphism. Thus this heat-stable ALPase appears to represent the expression of the human placental ALPase locus in lung. Until recently it was generally thought that human placental ALPase is expressed in the normal individual only in placenta. Its occasional "ectopic" appearance in certain malignancies of adult tissues has, however, long been recognized (22–24). We recently reported the occurrence of

Table 2. Inhibition of heat-stable ALPase in lung from four Old World monkeys and man

	$[I_{50}]$, mM									
	Phe		Har		Phe-Gly-Gly		Leva		Leu	
Species	*m*	SD	*m*	SD	*m*	SD	*m*	SD	*m*	SD
Baboon	11.4	—	>50	—	0.72	—	4.5	—	>50	—
Pig-tailed macaque	11.8	—	>50	—	0.77	—	4.9	—	50	—
Java monkey	15.6	—	>50	—	0.82	—	6.6	—	45	—
Rhesus monkey	17.1	—	>50	—	0.86	—	5.9	—	>50	—
Old World monkeys (combined $n = 4$)	14.0	2.8	>50	—	0.79	0.06	5.5	1.0	>50	—
Human ($n = 11$)	0.9	0.1	>50	—	0.16	0.08	1.1	0.5	5.4	2.1

human placental-type ALPase in nonmalignant adult cervix (10). The present findings show that nonmalignant lung is another tissue in which this ALPase appears in detectable, though very low, amounts in adults. The quantity of this placental ALPase present in lung and cervix amounts to only about 0.5% of that found in term placenta.

The very small amounts of human placental-type ALPase cannot be identified by standard inhibition and immunologic studies on unheated lung extracts because its presence is masked by the liver/bone/kidney-type ALPase, which composes most (96.5%) of the total ALPase activity. In order to find out if trace expression of either the heat-stable human placental-type ALPase or the heat-stable Old World monkey lung-type of ALPase occurs in other primate tissues, we carried out a systematic search for such heat-stable ALPase. The approach was to heat the tissue extract at 65°C for 1 hr, separate the insoluble material by centrifugation, and then concentrate the supernatant at least 10-fold. We could then determine the quantity of any heat-stable residual activity and, if this was sufficient in amount, characterize it by using our standard inhibition and immunological procedures. We found that to carry out these procedures we required at least 0.01 international unit/ml. This would on average represent at least 0.2% of initial activity and indicates the average lower limit of sensitivity of the method, though the sensitivity clearly varies with variations in total ALPase activity from one tissue sample to another and with the actual amount of tissue available for study. We have examined in this way liver samples from 19 species (chimpanzee, gorilla, gibbon, orangutan, mangabey, African green monkey, Celebese black ape, Java monkey, rhesus monkey, pig-tailed macaque, bonnet monkey, mandrill, baboon, owl monkey, marmoset, capuchin, tamarin, squirrel monkey, and woolly monkey), kidney samples from 15 species (chimpanzee, gorilla, orangutan, mangabey, Java monkey, rhesus monkey, pig-tailed macaque, barbary ape, baboon, owl monkey, marmoset, capuchin, woolly monkey, tamarin, and squirrel monkey), placental samples from 4 species [gibbon, owl monkey, rhesus monkey (11 samples), and pig-tailed macaque (3 samples)], and intestinal samples from 10 species (baboon, chimpanzee, pig-tailed macaque, bonnet monkey, owl monkey, marmoset, capuchin, tamarin, rhesus monkey, and squirrel monkey). In a few of the intestinal samples small amounts of residual activity were found, but on inhibition analysis this was shown to have the same inhibition profile as the ordinary intestinal ALPase of that species. In other cases any residual activity detected was below the limits of sensitivity of the inhibition methods. Thus if either human placental-type ALPase or Old World monkey lung-type ALPase occurs in these tissues it must be present in very small amounts.

EVOLUTIONARY CONSIDERATIONS

It has been suggested that the liver/bone/kidney, intestinal, and human placental-type ALPases represent a so-called multilocus enzyme system, the loci coding for the different isozymes having been derived in the course of evolution from a common ancestral gene as a result of successive duplications (25). Point mutations occurring in the genes subsequent to duplication would have led to divergence in the base sequences of their coding regions and hence to characteristic differences in the structures and properties of the ALPases they now determine. Furthermore, we have argued that in cases such as the human placental and intestinal ALPase that show marked differences of expression in different tissues, there must also have been an evolution of whatever genetic elements there are that regulate the expression of the loci in different tissues, and this must have occurred *pari passu* with the divergence in structure and properties of the enzyme products of the duplicated loci.

Our results suggest that expression of the characteristic human placental-type ALPase in placenta appeared relatively recently on the evolutionary scene: sometime after the divergence of the evolutionary lineages leading to man and the great apes (chimpanzee and orangutan) from the lineages leading to other primates, including gibbon, which is classified with man and the great apes in the Hominoideae, but in a separate taxonomic category. This finding can in principle be accounted for in terms of two rather different hypotheses. One is that the duplication from which the human placental-type ALPase originated occurred at this point in evolution. If so, it is necessary to assume that as the enzyme evolved structurally to give rise to the form now seen in man and the great apes, there was also occurring an evolution of a regulatory system that led to its major expression in placenta and very minor expression in other tissues such as lung and cervix. It is also necessary to suppose that the locus coding for the unusual ALPase found in large amounts in lung of Old World monkeys is the product of an earlier duplication, probably also occurring in primate evolution because we have not found this ALPase in New World monkeys or lower mammals. Again, concomitant evolution of a regulatory system leading to its major expression in lung but not other tissues would need to be postulated. The second hypothesis is that the locus determining human placental type ALPase arose by duplication much earlier but it did not become expressed as the major ALPase in placenta until after the divergence of the lineages leading to man and the great apes from the lineages leading to other primates. The evolutionary events concerned would thus involve mutation or mutations in regulatory systems resulting in derepression or activation in placenta of the previously unexpressed locus. In this view the ALPase in lungs of Old World monkeys could be the product of the same locus that determines the human placental ALPase, to which it is closely related immunologically. The structural differences between the two forms reflected in the marked differences in inhibition profile might be explained by divergence due to point mutations. Alternatively, the locus expressed in Old World monkey lung may, as in the first hypothesis, represent the end product of a separate duplication. A full resolution of these various questions will no doubt require direct studies on DNA by using the techniques of cloning, restriction endonuclease analysis, and DNA sequence analysis that have proved so successful in elucidating such multilocus systems as those that determine hemoglobin. This approach awaits the construction of suitable DNA probes.

We thank the following for sending us generous gifts of primate tissues: Elizabeth Muchmore and Alvie Gerlt (New York University Medical Center, Tuxedo, NY), Thea Sutherland and Judith Johnson (Regional Primate Research Center at the University of Washington, Seattle, WA) (National Institutes of Health Grant RR00166), Gisela Epple (Monell Chemical Senses Center, Philadelphia, PA), David Burr (Anatomy Department, Kansas University Medical Center, Kansas City, KS) (Grant KUMC 1514), Harold M. McClure and Ellen Lockwood (Yerkes Regional Primate Research Center, Emory University, Atlanta, GA), James Ebert and Charles Schable (Department of Health, Education, and Welfare, Center for Disease Control, Phoenix, AZ), Immunofluorescent Testing Service (Buffalo, NY), W. Ann Reynolds and Anne F. Bauman (Department of Anatomy, University of Illinois College of Medicine, Chicago, IL), Alan Laties (Department of Ophthalmology, University of Pennsylvania, Philadelphia, PA), D. P. Martin (Litton Bionetics, Kensington, MD), Gail Watts (Animal Hospital, Brookfield Zoo, Brookfield, IL), Howell Hood (Phoenix Zoo, Phoenix, AZ), Robert Snyder (Philadelphia Zoo, Philadelphia, PA), Jo Fritz (Primate Foundation, Tempe, AZ), South American Primate Research Center, Miami, FL), Janet Delinger (Columbia University College of Physicians and Surgeons, New York, NY), Daniel Dalgard (Hazelton Laboratories, Department of Primatology, Vienna, VA), and Zbigniew

Czernicki (Department of Neurosurgery, Hospital of the University of Pennsylvania, Philadelphia, PA). We also thank Vincent Stanton for some of the Ouchterlony studies. This work was supported by National Institutes of Health Grants GM 27018 and GM 07511.

1. Mulivor, R. A., Plotkin, L. I. & Harris, H. (1978) *Ann. Hum. Genet.* **42,** 1–13.
2. Mulivor, R. A., Hannig, V. L. & Harris, H. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3909–3912.
3. Seargeant, L. E. & Stinson, R. A. (1979) *Nature (London)* **281,** 152–154.
4. McKenna, M. J., Hamilton, T. A. & Sussman, H. H. (1979) *Biochem. J.* **181,** 67–73.
5. Goldstein, D. J., Rogers, C. E. & Harris, H. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2857–2860.
6. Doellgast, G. J. & Benirschke, K. (1979) *Nature (London)* **280,** 601–602.
7. Chang, C., Anstiss, C. L., Angellis, D. & Fishman, W. H. (1979) *Immunol. Commun.* **8,** 563–579.
8. Goldstein, D. J. & Harris, H. (1979) *Nature (London)* **280,** 602–605.
9. Fishman, W. H. & Sie, H.-G. (1970) *Clin. Chim. Acta* **29,** 330–341.
10. Goldstein, D. J., Blasco, L. & Harris, H. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 4226–4228.
11. Neale, F. C., Clubb, J. S., Hotchkis, D. & Posen, S. (1965) *J. Clin. Pathol.* **18,** 359–363.
12. Moss, D. W. (1975) *Enzyme* **20,** 20–34.
13. Khattab, M. & Pfleiderer, G. (1976) *Hoppe-Seyler's Physiol. Chem.* **357,** 377–391.
14. Lehmann, F.-G. (1975) *Clin. Chim. Acta* **65,** 257–269.
15. Fishman, W. H., Green, S. & Inglis, N. I. (1963) *Nature (London)* **198,** 685.
16. Fishman, W. H., Inglis, N. I., Sarke, F. & Ghosh, N. K. (1966) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **25,** 748 (abstr.).
17. Fishman, W. H. & Sie, H.-G. (1971) *Enzymologia* **41,** 141–167.
18. Lin, C.-W. & Fishman, W. H. (1972) *J. Biol. Chem.* **247,** 3082–3087.
19. Doellgast, G. J. & Fishman, W. H. (1977) *Clin. Chim. Acta* **75,** 449–454.
20. Van Belle, H. (1976) *Clin. Chem.* **22,** 972–976.
21. Doellgast, G. J. & Fishman, W. H. (1976) *Nature (London)* **259,** 49.
22. Fishman, W. H., Inglis, N. R., Green, S., Anstiss, C. L., Ghosh, N. K., Reif, A. E., Rustigan, R., Krant, M. J. & Stolbach, L. L. (1968) *Nature (London)* **219,** 697–699.
23. Stolbach, L. L., Krant, M. J. & Fishman, W. J. (1969) *N. Engl. J. Med.* **281,** 757–762.
24. Nathanson, L. & Fishman, W. H. (1971) *Cancer* **27,** 1388.
25. Harris, H. (1978) in *Genetics and Human Biology: Possibilities and Realities. Multilocus Enzymes in Man,* CIBA Found. Symp. No. 66, eds. Porter, R. & O'Connor, M. (Excerpta Medica, Amsterdam), pp. 187–199.