no curative effect of the vitamin. Humans with pernicious anemia respond to treatment with B_{12} by a marked increase in reticulocytes within 3 to 5 days (*4*). We found no increase in percentage of reticulocytes in either affected or control mice, 48 and 58 days old, at 5 days after the first of three daily injections of 3.75 μg of B_{12}. In affected growing mice, daily injections of 1 μg of B_{12} for 5 days per week, beginning at 2 or 4 days of age, failed to alleviate the anemia or the weight deficit. The treatment also failed to prevent the onset of behavioral symptoms at the usual time or to prevent early death.

As further evidence for lack of similarity between this syndrome and pernicious anemia, we have found that gastric acidity is normal (*p*H 1 to 2), and that the stomach is histologically normal in cribriform degeneration.

The distribution of Na^+, K^+, and Cl^- is abnormal in *cri/cri* mice. Observations (*5*) have shown that mutant mice drink physiological saline in preference to water, and that they have decreased concentration of K^+ in plasma, decreased concentrations of Na^+ and K^+ in urine, increased concentration of Na^+ in hair and feces, and increased concentration of Cl^- in the sweat on the plantar surface of the hind feet. With respect to electrolyte balance, *cri* has some similarity to cystic fibrosis, a recessively inherited disease in humans.

Other effects of *cri/cri* include a reduction in size of some of the endocrine glands. In particular, the anterior lobe of the hypophysis is disproportionately reduced in comparison with the other two lobes, the testes are small with fewer mature sperm and fewer interstitial cells than normal controls, and the thyroid has small follicles lined with epithelium that is lower in height than that of normal controls. These effects may be nonspecific consequences of the general debility of the affected animals. Electron microscopic examination of the anterior hypophysis of *cri/cri* mice shows growth hormone cells of normal appearance, as well as the other cell types seen in controls. In addition, although the vaginas of most *cri/cri* females do not open even if they survive 20 days or more beyond the normal time of vaginal opening (about 30 days in DBA/2J controls), vaginal opening was found in one fairly healthy affected female at 54 days, and was followed by a normal estrous cycle 10 days later.

The fur and vibrissae of most *cri/cri* mice are slightly wavy. This is true both on the DBA/2J background and in affected mice from the linkage cross. This characteristic is often helpful in identifying affected mice before the abnormal behavior becomes severe enough to be diagnostic.

The basic defect caused by *cri* is as yet unknown. The abnormal distribution of electrolytes suggests that an abnormality of electrolyte transport may underlie the other defects, but the mechanism remains to be discovered.

MARGARET C. GREEN
Jackson Laboratory,
Bar Harbor, Maine 04609
RICHARD L. SIDMAN
Department of Neuropathology,
Harvard Medical School,
Boston, Massachusetts 02115
OMAR H. PIVETTA
Jackson Laboratory

References and Notes

1. R. L. Sidman, J. B. Angevine, E. Taber Pierce, *Atlas of the Mouse Brain and Spinal Cord* (Harvard Univ. Press, Cambridge, Mass. 1971).
2. L. Van Bogaert and I. Bertrand, *Acta Neurol. Psychiatr. Belg.* **49**, 572 (1949); B. Q. Banker, J. T. Robertson, M. Victor, *Neurology* **14**, 981 (1964); G. R. Hogan and E. P. Richardson, Jr., *Pediatrics* **35**, 284 (1965); M. Adachi, B. J. Wallace, L. Schneck, B. W. Volk, *J. Neuropathol. Exp. Neurol.* **25**, 598 (1966).
3. R. L. Chandler, *Brit. J. Exp. Pathol.* **49**, 52 (1968); P. Lampert, K. Earle, C. J. Gibbs, D. C. Gajdusek, *J. Neuropathol. Exp. Neurol.* **28**, 353 (1969); P. W. Lampert, D. C. Gajdusek, C. J. Gibbs, *ibid.* **30**, 20 (1971).
4. R. B. Thompson, *Hematology* (Lippincott, Philadelphia, 1961), pp. 132–135.
5. O. H. Pivetta and M. C. Green, in preparation.
6. Supported in part by NIH grants NS 09378, NS 08359, and AM 15587; NSF grant GB 27487; and a grant from the Cystic Fibrosis Research Foundation. We thank Drs. E. M. Eicher, E. S. Russell, and C. Waymouth for the use of their facilities, Ruth T. Farnsworth, Janice L. Southard, Eleanor C. McFarland, Catherine K. Suiter, Hope O. Sweet, Ruth Trotzer, Diane Gaudreau, and Pieter Dikkes for technical assistance. The Jackson Laboratory is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

14 February 1972; revised 20 March 1972

Primate Phylogeny and Immunological Distance

Abstract. *Recalculation of the time of divergence of the Pongidae and Hominidae after correction of immunological distance by inclusion of generation length yields minimum dates of approximately 14 million years ago.*

Estimates of the temporal separation of the Hominidae and Pongidae have been the source of heated debate for over a century. Calculations based upon immunological distances of homologous serum proteins have been used to challenge more traditional approaches, which usually place the age of this division sometime in the Miocene (*1–4*). The strongest advocates of the "protein clock" approach have suggested dates as recent as 4 million to 6 million years ago (*5*, *6*). Their calculations are based upon two fundamental assumptions. The first is that some significant proportion of amino acid residues in the primary structure of a protein are subject to replacement by other residues without a significant alteration of the biological function of the protein. The second is that such replacements occur at rates that are subject to mensuration in units of geologic time (*7*, *8*). We believe that the latter may involve a fundamental error.

For either divergence times or "rates" of protein evolution to be determined, mutation rates and rates of amino acid substitutions must be calibrated in biologically meaningful units of time, such as nucleotide shifts (point mutations) per DNA replication per standard time unit. Mitosis provides an opportunity for nucleotide altera-

Table 1. Data for DNA hybridization and albumin immunological distances, ID, for various primates with calculated mutation rates, μ, in units of ID per generation per million years. The DNA hybridization distances were obtained by reassociation of radioactive nonrepeated DNA with total nonradioactive DNA, followed by assay of thermal stability (*10*). The albumin immunological distances were obtained by micro-complement fixation (*6*).

Primates	DNA		Serum albumin	
	Nucleotide change since divergence (%)	μ	ID	μ
Man–chimpanzee	2.6*		7*	
Man–prosimian†	54	1.19	123	2.71
Old World monkey–chimpanzee	10.8	0.98	30	2.72

* Used to calculate α. † The primate used for DNA study was the *Galago*. The albumin value is an average for *Lemur* and *Loris*.

tions once per cell generation. Thus, the mutation rate will be dependent upon the number of cell generations that separate zygote and gamete. This is undetermined for mammals (*9*) but, because primordial germ cells are set aside early in the embryo, there is no basis at present which indicates that the number of cell generations involved substantially varies in primates of different generation lengths. Therefore, a factor that critically affects the time rates of protein evolution is generation length (*10, 11*). The inclusion of generation length has a dramatic effect upon biochemically determined dates of the hominid-pongid divergence. As we are primarily concerned with illustrating the effect of its inclusion in calculations of protein evolutionary rates, we have used only one definition here (150 percent the age of menarche) (*12*).

A major trend in Cenozoic primate evolution has been the progressive neocortical encephalization (*13–15*). A number of reproductive adaptations have accompanied this increase; these include greater gestation periods, increased "efficiency" of placentation, and longer generation lengths (*11, 13, 14, 16*). Because these adaptations are highly correlated with the degree of encephalization among living primate taxa (*13, 14, 17*), we have used the development of the neocortex (as judged from fossil endocasts) as an index of generation length in extinct taxa.

The similarity of encephalization between living prosimians and Paleocene and Eocene primates has been pointed out (*13, 18*). We have thus estimated generation length in the Paleocene on that of *Tupaia* (*19, 20*), and in the Eocene on those of lemuriforms and lorisiforms, for which observations of age of menarche are available (*21, 22*). We have made a third estimate for 30 million years ago, which is the approximate K/Ar determination for *Aegyptopithecus* (*23, 24*). The cranial anatomy of this form is generally similar to that of extant cercopithecoids (*24*), and we have therefore estimated the age of menarche in the hominoids of this epoch by that of present-day Old World monkeys (*25*). A final point is based upon data for the modern chimpanzee (*26*). The number of generations per year for these four points has been plotted in Fig. 1. A curve of the form $X_1(t) = at^b$ was fitted to these points (see legend to Fig. 1).

We also make the assumption that

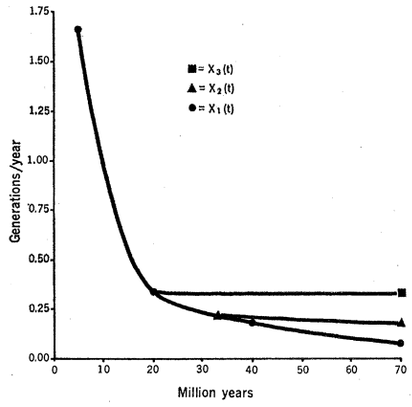

Fig. 1. Primate generations per year [$X_1(t)$] plotted against years since the beginning of the Cenozoic. The $X_1(t)$ have been approximated by $X_1(t) = 10.75t^{-1.153}$ ($r = -.9989$). The number of generations per year of the Old World monkey is assumed to be of the form $X_2(t) = \bar{a}t^{\bar{b}}$, where the values $X_2(33) = X_1(33) = 0.191$ and $X_2(70) = 0.167$ are known. This gives $X_2(t) = 0.216t^{-0.181}$. The number of generations per year of *Lemur* and *Loris* are assumed to be $X_3(t) = X_1(t)$ for t between 5 million and 20 million years and $X_3(t) = 0.333$ for t between 20 million and 70 million years.

generation length in the hominid and pongid clades (phyletic lines) has not substantially differed for the greater part of their existence as separate lineages. That is, we assume that the marked encephalization of hominids (and commensurate increase in generation length) was restricted to the late Pliocene and Pleistocene. If we also assume that immunological distances (ID), or similar data such as DNA hybridization data (see Table 1), are functions of the number of generations occurring in both lineages since their divergence, we can find the divergence dates (α) by summing the accumulated ID as follows:

$$\mathrm{ID}_{\mathrm{C\text{-}M}} = 2\mu \int_{\alpha}^{70} X_1(t)\,dt$$

where $\mathrm{ID}_{\mathrm{C\text{-}M}}$ is the immunological (or DNA) distance between chimpanzee and man, and μ is the mutation rate in ID units per generation per million years. We can find the mutation rate (μ) by considering the divergence dates of hominoids and other primates from:

$$\mu = \frac{\mathrm{ID}_{\mathrm{H\text{-}Y}}}{\int_{\beta}^{70} [X_1(t) + X_j(t)]\,dt}$$

where $\mathrm{ID}_{\mathrm{H\text{-}Y}}$ is the immunological distance for a hominoid and other primate lineage, β is the divergence date for the lineage, $j = 3$ for ID's between man and prosimians, and $j = 2$ for ID's between chimpanzees and Old World monkeys (see legend of Fig. 1).

Assuming that the populations ancestral to the Hominoidea separated from those ancestral to the Cercopithecoidea about 37 million years ago (Eocene-Oligocene boundary) and that those ancestral to the Lemuriformes and Lorisiformes separated from the Hominoidea about 65 million years ago, we calculated mutation rates and times of divergence for man and chimpanzee from the ID's in Table 1. The solutions gave divergence dates of 14 million years ago from albumin ID's and 14 million and 12 million years ago from DNA hybridization percentages.

The above results should be regarded as minimum estimates. Even if the assumption of equivalent generation length for hominids and pongids is correct, there still remain the prolonged generations of hominids in the late Pliocene and Pleistocene. In addition, we have consistently chosen conservative calibration dates. It is likely that the divergence of the hominoids and cercopithecoids took place before the Eocene-Oligocene boundary and that the divergence of anthropoids and lemuriforms and lorisiforms took place before the Paleocene-Eocene boundary (*27*). On the other hand, if the relatively long generations of the Hominoidea are a very recent phenomenon, then we may have calculated too early a divergence. At the present, however, this does not seem likely; this brings us to the final and most important consideration.

Specific dates of divergence for hominids and pongids based upon immunological distances among primates have received considerable criticism on grounds other than those discussed in this report (*8, 28*). These criticisms, in addition to the dramatic effect of normalization for generation length, demonstrate the equivocal accuracy of the "protein clock." The calculations presented above require numerous assumptions, any one of which, if significantly incorrect, would lead to substantial error. The "protein clock," when properly calibrated, requires the assumption of constant rates of protein evolution (and ignores such phenomena as parallel evolution at the molecular level), requires estimates of other divergence dates for calibration, requires accurately estimated generation lengths, and requires the assumption (or calculation) of the number of cell gener-

ations between zygote and gamete. Thus, our conclusions point to the need for even more detailed study of comparative anatomy and the fossil record, because without such study, the accurate calibration of "protein clocks" will be difficult if not impossible.

C. OWEN LOVEJOY
Department of Anthropology,
Kent State University,
Kent, Ohio 44242

ALBERT H. BURSTEIN
KINGSBURY G. HEIPLE
Biomechanics Laboratory,
Division of Orthopaedic Surgery,
Case Western Reserve University,
Cleveland, Ohio 44106

References and Notes

1. B. G. Campbell, *Human Evolution* (Aldine, Chicago, 1966); B. Kurten, in *Evolution und Hominisation*, G. Kurth, Ed. (Verlag, Stuttgart, 1968), p. 75; A. H. Schultz, in *Ideas on Human Evolution*, W. Howells, Ed. (Atheneum, New York, 1967), p. 36; E. L. Simons and D. R. Pilbeam, *Folia Primatol.* **3**, 81 (1965).
2. E. L. Simons, in *Evolutionary and Genetic Biology of Primates*, J. Buettner-Janusch, Ed. (Academic Press, New York, 1963), vol. 1, p. 66.
3. ———, *Proc. Nat. Acad. Sci. U.S.* **51**, 528 (1964).
4. R. H. Tuttle, *Science* **166**, 953 (1969).
5. V. M. Sarich and A. C. Wilson, *ibid.* **158**, 1200 (19[illegible] A. C. Wilson and V. M. Sarich, *Proc. Nat. Acad. Sci. U.S.* **63**, 1088 (1969); V. M. Sarich, in *Perspectives on Human Evolution*, S. L. Washburn and P. C. Jay, Eds. (Holt, Rinehart & Winston, New York, 1968), p. 94.
6. V. M. Sarich, in *Background for Man*, P. Dolhinow and V. M. Sarich, Eds. (Little, Brown, Boston, 1971), p. 60.
7. ——— and A. C. Wilson, *Science* **154**, 1563 (19[illegible] *Proc. Nat. Acad. Sci. U.S.* **58**, 142 (1967); V. M. Sarich, in *Old World Monkeys*, J. R. Napier and P. H. Napier, Eds. (Academic Press, New York, 1970), p. 175.
8. D. W. Read and P. E. Lestrel, *Science* **168**, 578 (1970).
9. F. Vogel, in *Genetics Today: Proceedings of the 11th International Congress of Genetics*, S. Geerts, Ed. (Pergamon, London, 1965), p. 833.
10. D. E. Kohne, J. A. Chiscon, B. H. Hoyer, *Carnegie Inst. Washington Yearb.* **69**, 488 (1971).
11. M. Goodman, *Hum. Biol.* **34**, 104 (1962).
12. The important variable here is the age of menarche. Other percentage functions do not measurably alter the results.
13. W. E. Le Gros Clark, *Antecedents of Man* (Harper, New York, 1959).
14. A. H. Schultz, *Life of Primates* (Universe, New York, 1969).
15. W. C. O. Hill, *Man's Ancestry* (Heinemann, London, 1954).
16. M. Goodman, in *Classification and Human Evolution*, S. L. Washburn, Ed. (Aldine, Chicago, 1963), p. 204.
17. J. Buettner-Janusch, *Trans. N.Y. Acad. Sci.* **31**, 128 (1969); H. Stephan and O. J. Andy, *Ann. N.Y. Acad. Sci.* **167**, 370 (1969); C. R. Noback and N. Moskowitz, *ibid.* **102**, 210 (1962).
18. E. L. Simons, *Ann. N.Y. Acad. Sci.* **102**, 282 (1962); L. B. Radinsky, *Amer. J. Phys. Anthropol.* **27**, 385 (1967); W. E. Le Gros Clark, *J. Anat.* **79**, 123 (1945); C. L. Gazin, *Smithson. Misc. Collect.* **149**, 1 (1965); W. E. Le Gros Clark and D. P. Thomas, *Fossil Mammals of Africa* (No. 5) (British Museum, London, 1952).
19. We have used an estimate of 143 days as the age of menarche; this is the average of values given in (*14*) and (*20*).
20. J. R. Napier and P. H. Napier, *Handbook of Living Primates* (Academic Press, New York, 1967).
21. An average estimate is 2 years, based on data from (*22*) for *Lemur* and *Propithecus* and from (*20*) for *Lemur*, *Lepilemur*, and *Microcebus*.
22. A. Jolly, *Lemur Behavior* (Univ. of Chicago Press, Chicago, 1966).
23. E. L. Simons, *Nature* **205**, 135 (1965).
24. ———, *Sci. Amer.* **217**, 28 (1967); *Amer. Mus. Nov.* (No. 1976), 1 (1959).
25. We have used an estimate of about 4 years, the average of estimates for *Macaca*, *Papio*, and *Prysbytis* (*20*).
26. The estimate used was 8.8 years (*20*).
27. E. L. Simons, *Ann. N.Y. Acad. Sci.* **167**, 319 (1969).
28. T. Uzzell and D. Pilbeam, *Evolution* **25**, 615 (1971).

16 December 1971; revised 8 March 1972

Insulin Receptor of Fat Cells in Insulin-Resistant Metabolic States

Abstract. *A diminished response to insulin is exhibited by isolated fat cells obtained from rats that have been either starved, or treated with prednisone, or made diabetic by administration of streptozotocin. This decrease in response is not accompanied by changes in the quantity of insulin receptor of these cells or in the affinity of these receptors for insulin. Similarly, the decreased responsiveness to insulin of fat cells obtained from certain species (hamster, rabbit, mouse, guinea pig) is not explainable in terms of alterations of the insulin receptor.*

Insulin exerts many, if not all, of its metabolic effects on adipose cells by interacting with specific superficial receptors on the membrane (*1*, *2*). The specific binding of [^{125}I]insulin to intact, metabolically responsive cells appears to provide a sensitive method of directly measuring the initial interaction of the hormone with specific cell receptors by means which are independent of complex metabolic parameters (*3*). This binding interaction with intact cells and with cell membranes involves biologically active receptors for insulin (*3*, *4*). We studied the specific binding of [^{125}I]insulin to isolated adiposites of rats in a variety of "insulin-resistant" metabolic states in order to assess the possible contribution of defective or diminished receptor binding in these conditions.

Adiposites obtained from rats that

Table 1. Effect of starvation, prednisone administration, streptozotocin diabetes, and species differences on insulin enhancement of glucose transport in isolated adiposites. Adiposites were prepared (*11*) from epididymal fat pads of Sprague-Dawley rats. The cells (0.5×10^4 to 2×10^4) were incubated for 90 minutes at 37°C in 1 ml of buffer (Krebs-Ringer-bicarbonate) containing 2 percent (weight to volume) albumin and 0.2 m*M* D-[^{14}C]glucose (5.1 μc/μmole) (*2*). Rats treated with prednisone received 1 mg of prednisone intraperitoneally daily for 7 days. Streptozotocin was administered as a single intraperitoneal injection in 0.1*M* sodium citrate buffer, *p*H 4.0, at a dosage of 70 mg/kg, 7 days prior to the experiment. The diabetic state was indicated by a glucosuria greater than 0.5 mg per 100 ml. Separate experiments, each with separate control cells, are also presented. Various concentrations of porcine insulin were used in the range of 10^{-11} to 10^{-8}*M*; only representative values are presented here. The glucose oxidation assay was repeated at least once for each "resistant" state. Standard deviations in these experiments varied from 3 to 6 percent. The specific binding of [^{125}I]insulin to fat cells from the different animals is described in Table 2.

Animal	Weight (g)	Conversion of D-[^{14}C]glucose to $^{14}CO_2$ [moles of $^{14}CO_2$ per cell per minute ($\times 10^{16}$)] at insulin concentrations of:				
		0	8.5×10^{-11}*M*	5.3×10^{-9}*M*	2.6×10^{-8}*M*	Increase* (%)
Fed rat†	320	3.3		15		354
Fed rat†	120	3.0		12		300
Starved rat‡	230	1.0		1.2		20
Control rat	133	2.2	7.9		9.5	332
Prednisone-treated rat	101	1.6	2.0		2.9	81
Control rat	137	5.3	16		17	220
Streptozotocin-diabetic rat	138	3.4	4.6		5.2	53
Control rat	101	1.8	7.7		9.7	438
Hamster	107	1.6	1.5		1.7	6
Guinea pig	457	3.0	3.3		3.7	23
Control rat	127	5.9	9.8		13	120
Mouse	27	7.4	7.8		8.6	16
Rabbit	3000	2.8	2.9		2.0	

* Increase of baseline rate of glucose oxidation observed with the highest insulin concentration studied. † Average diameter of fat cells from 320-g rats, 70 μm; average diameter of cells from 120-g rats, 32 μm (*10*). ‡ Obtained from the control, fed group (320 g), then starved for 5 days; average fat cell diameter, 36 μm.