monoploids among the 4078 embryos that occurred singly ($P = .11$). In the 1968 androgenetic selections (*ig ig* and *Ig ig* females) there were five among the 316 plural embryos and 70 among the 8975 single embryos ($P = .12$). The weak association indicated is accounted for by a unique minority class —four of the 12 polyembryonic monoploids had as their R^{nj} twin an embryo with 10 chromosomes. These four cases of gynogenetic-androgenetic twins are to be compared with an expectation of 0.07 based on random occurrence. The gynogenetic-androgenetic coincidence is understandable if *ig* expression is variable from one embryo sac to another, and if common conditions promote parthenogenetic development of cells possessing nuclei derived from either kind of gametophyte.

Action of the *indeterminate gametophyte* gene reflects the loss of normal functions in female gametophyte development. Ordinarily, only a given number of specialized cells are produced. The egg and polar nuclei in the normal, mature gametophyte may be considered mitotically arrested. The pleiotropic effects of *ig* are understandable if *ig* releases the embryo sac from this mitotic restraint. Additional polar nuclei and eggs form, and so predispose to elevation of endosperm ploidy and polyembryony. The response of a sperm nucleus to an *ig* embryo sac is not inconsistent with this view. Since *Ig* and *ig* male gametophyte nuclei behave similarly, embryonic development of sperm appears normally to be repressed by action of *Ig* in the female gametophyte. Under *ig* influence, cells of the embryo sac may proliferate, yielding additional gametophyte cells, whereas sperm in the environment of the embryo sac develop embryonically rather than produce additional male gamete nuclei. This would account for the differential increase of androgenesis as compared to gynogenesis.

Androgenesis incited by *ig* may provide a more general method of generating monoploids, useful for example in cytogenetic investigations or in gamete selection programs of breeding, than available methods based on gynogenesis. The principal unknown factor in evaluating the *ig* system for this purpose is the extent to which the frequency of androgenesis is influenced by the residual genotype of the sperm.

Also, *ig* may be useful in the study of extranuclear heredity. If androgenesis involves the development of a sperm nucleus in maternal cytoplasm, the net result is equivalent to cytoplasmic substitution by nuclear transplantation. Three instances of spontaneous androgenesis observed following crosses of standard males to females carrying cytoplasmic male sterility bear on this question. The maternal descendants of one plant were uniformly male sterile (*11*), some descendants of the second were partially fertile (*4*), and most of the descendants in the third case were fully fertile (*12*). The second case, involving partial fertility, initially was considered to reflect segregation for fertility-restoring nuclear factors, even though restoration was not anticipated based on previous experience with these parents. With discovery of the third case it was suggested that fertility may result from transmission of male cytoplasm with the subsequent sorting out of cytoplasmic types. Seemingly, therefore, a given case of androgenesis may involve either substitution or hybridization of cytoplasms. The latter phenomenon, in conjunction with *ig* induced androgenesis, suggests a basis for the genetic analysis of extranuclear determinants in maize.

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14. I thank B. Lin for determinations of chromosome number and R. A. Brink and R. I. DeMars for suggesting changes in the manuscript. Paper No. 1331 of the Laboratory of Genetics.

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Tobacco Smoke Toxicity: Loss of Human Oral Leukocyte Function and Fluid-Cell Metabolism

Abstract. *The human mouth has been utilized as a new and significant in vivo open bioassay system for tracing undesirable substances present in tobacco smoke, in an exact milieu where the smoking "problem" begins and must be dealt with directly. The associated in vitro closed test systems described herein have provided new, sensitive bioassays that help to explain the in vivo effects.*

The human oral cavity can be utilized as an open test system to provide: (i) substantial numbers of functional oral leukocytes (inflammatory cells), (ii) actively metabolizing oral fluid-cell harvests (*1–3*), and (iii) a natural "smoking machine" and trap for tobacco smoke, based on the fact that the oral cavity is the portal of entry for this smoke into the human organism. This test system provides a useful supplement to observations on biological effects of tobacco smoke gained from use of other assay systems such as clam cilia (*4*); rodent, cat, dog, chicken, and monkey trachea (*5*); paramecia (*6*); rodent macrophages (*7*); and mouse skin painting and other similar techniques (*8*) (also see *9–12*).

We were interested in the effects of tobacco smoke immediately after a human subject puffed a cigarette. In a survey of more than 80 individuals (smokers and nonsmokers), we found that the number of puffs per cigarette varied from 6 to 27. Some smokers retained each puff 1 or 2 seconds longer than the average smoker, who took between 7 to 10 puffs into his month, retaining each puff for approximately 2 seconds. If a smoker does not inhale or swallow any of the smoke, the oral cavity is exposed repetitively to whole tobacco smoke, or to its gas phase (provided

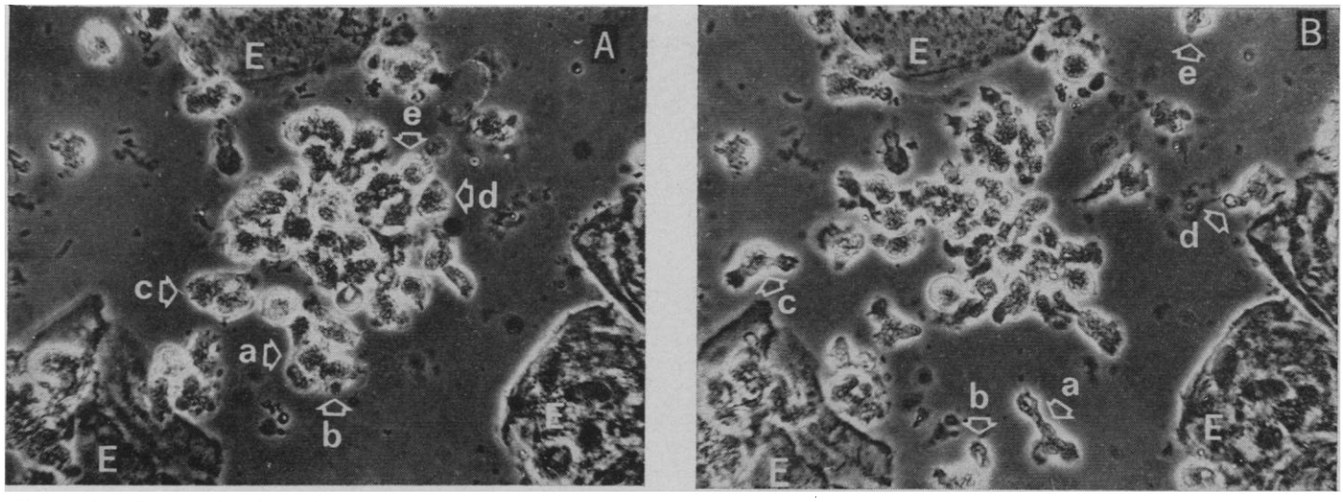

Fig. 1. (A) Surrounded by epithelial cells (*E*), a cluster of inflammatory cells from a control oral harvest displays signs of early activity. Note cells *a, b, c, d,* and *e.* Zeiss phase contrast microscope (× 315). (B) Forty minutes later, the inflammatory cells *a, b, c, d,* and *e* give an impression of the extent of locomotion when the position of these cells is compared to that occupied in (A) (× 315).

particulate matter is first removed experimentally from the smoke stream). That portion of whole tobacco smoke, or its gas phase, that remains in the mouth after several puffs of smoke are inhaled and exhaled through the mouth may be regarded as an oral retained residuum, which can be obtained essentially intact in a biological system. Our findings demonstrated that exposure of the mouth to whole tobacco smoke and its retained residuum (or to the particle-free gas phase and its retained residuum), after the subject had smoked one cigarette, provides enough toxic material to inhibit completely the function of the *in situ* exposed oral leukocytes. Exposure to whole tobacco smoke and its retained residuum also can induce 50 percent inhibition, or more, of the aerobic oxidative metabolism and anaerobic glucolysis of the *in situ* exposed oral fluid-cell components. All commercial cigarettes tested, including those with various filter tips, produce these evidently undesirable effects.

Apparently healthy human subjects (45 male and 41 female smokers and nonsmokers) were studied. A standardized method of sampling the oral cavity before and after smoking was established which employed balanced physiological rinse solutions. Equal amounts of dextran solution mixture (DSM) (*2*) and Krebs-Ringer bicarbonate buffer (KRB), *p*H 7.4, (or Krebs-Ringer phosphate buffer, *p*H 7.4, for aerobic metabolic studies) were combined and used as the harvesting medium. Chewing paraffin for 30 seconds in the presence of this physiological medium yielded oral fluid-cell harvests that contained millions of functional inflammatory cells capable of (i) pseudopodia formation, (ii) protoplasmic stretching and flow associated with organelle movement, (iii) locomotion, and (iv) phagocytosis as evidenced by engulfment of large and small microorganisms contained in the harvests. This method of sampling provided an effective milieu for virtually all subjects studied (*13*). To collect samples, any given subject first rinsed his or her mouth thoroughly with water; 10 minutes later, a first sample was taken with 5.0 ml of DSM and KRB; this was discarded. For the studies of leukocytes and anaerobic metabolism, a second sample was taken 45 minutes after the first sample and was labeled the 0- to 45-minute nonsmoking control. Forty-two minutes after the second sample was obtained, the subject began to smoke a cigarette (without talking, swallowing, or inhaling and exhaling through the nose), thus allowing the mouth to serve as a natural smoke trap. At precisely 45 minutes after the second sample, a third one, the 45- to 90-minute smoking sample, was obtained. For all metabolic studies, a fourth sample was collected, the 90- to 135-minute recovery control. All procedures were conducted at room temperature, except the respirometry assays for which the temperature was 37°C.

We have found that such oral fluid-cell harvests usually contain significant numbers of (i) peripheral leukocytes [approximately 75 percent polymorphonuclear neutrophils and 25 percent lymphocytes (*14*)]; (ii) sloughed epithelial cells; (iii) granular masses or casts; (iv) large rods, large filamentous forms, and numerous smaller microorganisms; and (v) large numbers of organelles and other bits of protoplasm derived from the host mammalian cells.

The functional activity of the leukocytes, mostly polymorphonuclear neutrophils, was recorded photographically in two ways—(i) sequential exposures taken every 2 minutes on 35-mm film during a 1-hour period (see Figs. 1 and 2), and (ii) time-lapse motion picture sequences taken during a 1- to 4-hour period.

Five to ten minutes were required to take a harvest, prepare a clot pellet and slide specimen, locate a suitable cluster of leukocytes, and take the first photomicrograph. In control samples, when the leukocytes were first observed, formation of active pseudopodia, protoplasmic flow, and locomotion were seen. The first photograph captures this early activity (Fig. 1A) and represents findings obtained for both smokers and nonsmokers, regardless of the brand of filter or nonfilter cigarette smoked. At the periphery of the field in this figure, portions of sloughed epithelial cells (*E*) can be discerned. Since the epithelial cells do not undergo any visible physical or cytological alteration during the time course followed herein, they serve as fixed landmarks for the evaluation of changes in the leukocytes, especially locomotion. A typical cluster of leukocytes is centered in the field in Fig. 1A. This cluster gives an impression of beginning to expand, as a result of the extension and stretching of most of the

Table 1. Aerobic endogenous and glucose-dependent metabolism of oral fluid-cell harvests before and after the smoking of one standard-brand cigarette. The subject, No. 29, was a 36-year-old male who took eight puffs per 3-minute smoking period. The body of the flask contained 1.0 ml of oral fluid-cell harvest. For endogenous activities, 2.0 ml of glucose-free dextran solution mixture plus Krebs-Ringer phosphate buffer (DSM + KSP), *p*H 7.4, was added; for glucose-dependent activities, 1.75 ml of DSM + KRP and 10 mg of D-glucose in 0.25 ml of DSM + KRP were added. The center well contained 0.25 ml of 10 percent NaOH. Temperature, 37°C; atmosphere, air.

Oral fluid-cell harvests	Aerobic metabolism (O_2 uptake)					
	Endogenous activity (μl)		Endogenous plus glucose activity (μl)		Glucose-dependent activity (μl)	
	1 hour	2 hours	1 hour	2 hours	1 hour	2 hours
0–45 minute control	43	69	82	169	39	100
45–90 minute sample after smoking	38	49	45	74	7	25
90–135 minute recovery control	27	40	48	92	21	52

individual inflammatory cells in an attempt to disengage from the cluster and to move radially in all directions. Various bacterial forms and granular masses, as well as organelles from disrupted host cells (mostly inflammatory in nature) are also in the field. In Fig. 1B the same cluster is shown after a 40-minute, on-the-slide incubation period. The degree and extent of locomotion that has occurred can be judged by comparing the position and shape of the leukocytes in Fig. 1B with those in Fig. 1A. Most of these cells are in a state of high functional activity and can readily engulf foreign matter and phagocytize bacteria, although the latter process is not readily apparent in this sequence.

Another cluster of leukocytes obtained from the same subject immediately after he smoked one cigarette is shown in Fig. 2A. This cluster is also positioned in the center of the field adjacent to a group of epithelial cells on the left. Most of the leukocytes are spherical in shape and appear paralyzed. In some cases, the leukocytes present after smoking are rounded and their cytoplasmic granules exhibit active Brownian motion. In other instances, some cells attempt to form pseudopodia and move about but form cytoplasmic vesicles instead, which are classical signs of cell injury. At times, an occasional inflammatory cell can move sluggishly, but atypically. Any one of these states may represent the dominant response to the smoking of a single cigarette, although several states may be present concomitantly. At the end of a 40-minute incubation period, the appearance of the cluster in Fig. 2B demonstrates that under the conditions of the experiment these cells cannot recover and remain incapable of overcoming the inhibition of locomotion and phagocytosis caused by the toxic substances left behind by tobacco smoke.

Like paraffin-stimulated whole salivas from humans (*1–3*), the oral fluid-cell harvests under study here (*15*) exhibit measurable (sometimes considerable) aerobic endogenous and glucose-dependent consumption of oxygen, aerobic and anaerobic glucolysis, and other enzyme and metabolic activities. We regard the metabolism of these harvests to be mixed because of the varied cytology of their contained, identifiable components. However, we have concluded that the bulk of the metabolism of the harvests is associated with their mammalian cell fractions and that they behave mostly like mammalian cell homogenates of probable leukocytic origin (*1–3, 15*).

The aerobic endogenous and glucose-dependent oxygen uptake and anaerobic glucolytic metabolism of oral fluid-cell harvests were inhibited in most subjects after smoking a single cigarette. Typical examples are given in Tables 1 and 2; conventional Warburg respirometry was used. Control experiments were performed in which the actual smoking of one cigarette was omitted. In most of the latter instances no inhibitions were observed.

The Cambridge CM 113A filter (*16, 17*), composed mostly of glass and asbestos fibers, if placed in a stream of tobacco smoke and air, can block 99.9 percent of the particulate phase of tobacco smoke which constitutes the "tars" and contains virtually all the nicotine, while freely permitting the passage of the volatile gas-vapor phase, because puff characteristics are essentially unaltered. In this manner tobacco smoke is separated into two main fractions. This filter was employed in another se-

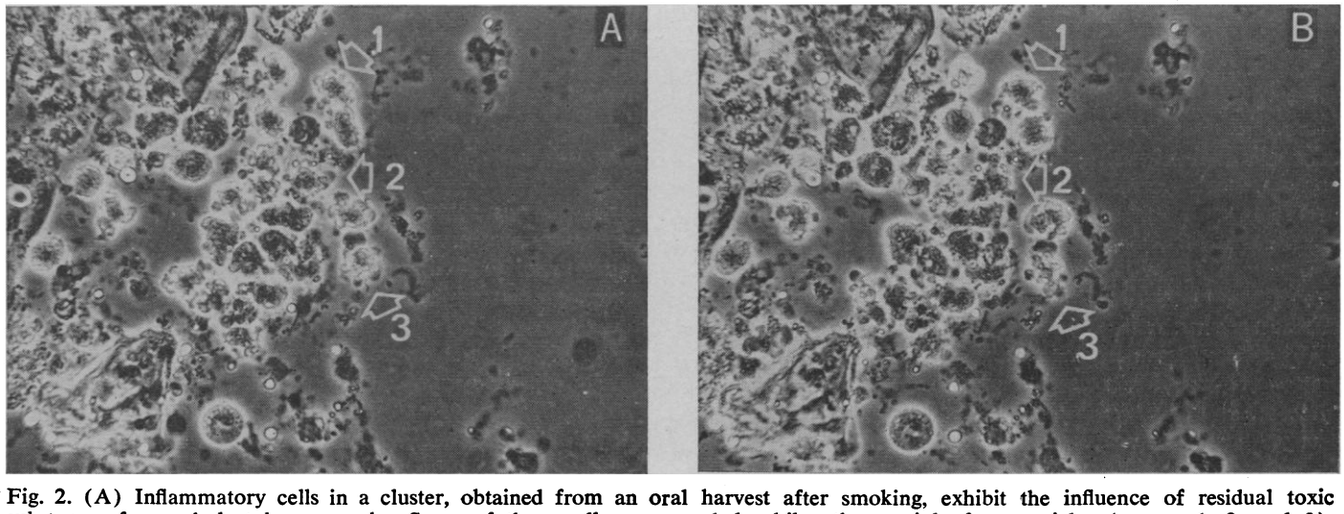

Fig. 2. (A) Inflammatory cells in a cluster, obtained from an oral harvest after smoking, exhibit the influence of residual toxic substances from whole tobacco smoke. Some of these cells are rounded, while others vainly form vesicles (arrows 1, 2, and 3). These cells are in the early stages of degeneration (× 315). (B) After a period of 40 minutes, the inflammatory cells shown in (A) have remained stationary except for sluggish movements associated with vesicle formation, which now occurs in many of the cells (× 315).

ries of experiments, which assured contact and interaction between the particle-free gas phase and the fluids and cells that coat the oral mucous membranes and the teeth. In every instance, regardless of subject or type of tobacco product smoked, the gas phase interfered with and inhibited inflammatory cell function in a manner similar to that seen for the whole tobacco smoke.

The relative toxicity of several known components of tobacco smoke was measured by use of the fluid-cell harvests from the human mouth in closed test systems in vitro. The two most potent substances of the gas phase found to date are acrolein and cyanide, which, at concentrations as low as $1 \times 10^{-6}M$ to $2.5 \times 10^{-6}M$ and $1 \times 10^{-5}M$ to $4.6 \times 10^{-5}M$, respectively, produce 50 to 100 percent inhibition of leukocyte function and of the metabolism of oral fluid-cell harvests. These concentrations of acrolein and cyanide are less than the concentrations in a single puff of tobacco smoke, yet they induce effects at least comparable to those elicited in the human open test system by the puffing of one cigarette.

In another series of experiments we have demonstrated that the function of oral leukocytes remains unimpaired under conditions that selectively exclude the toxic substances contained in the gas phase, but which permit significant amounts of the particle phase to enter the mouth. We, therefore, conclude that the volatile gaseous phase of tobacco smoke (not the "tars" or nicotine) contains most of the undesirable substances toxic to the human oral leukocyte.

Results of studies carried out in our laboratories with cigarettes currently manufactured and marketed in the United States, filter or nonfilter, indicate that the retained residuum of the gas-vapor phase of tobacco smoke from puffing a single cigarette is toxic to, and seriously impairs the described functions of, human oral leukocytes. Such an effect must be presumed to be undesirable since cells of this type constitute an essential line of body defense. Because oral leukocytes are secreted into the oral cavity in substantial numbers, and are continuously replaced by successors, the actual significance of these effects of smoke in terms of the health, well-being, and longevity of the host can only be determined by long-term observations or life-time experiments. The development of our present bioassay method should make possible the design of animal experiments in which chronic exposures to smokes with differing and controlled degrees of this type of biological activity are compared in regard to pathology, disease, or diminished life-span. The ultimate experiment of this type would involve use of a smoke devoid of the acute effects in question to see whether the end points mentioned were absent or diminished.

Table 2. Anaerobic glucolysis of oral fluid-cell harvests before and after the smoking of one standard brand cigarette. (Human oral fluid-cell harvests exhibit negligible endogenous glucolytic activities.) The subject, No. 4, was a 30-year-old male, who took nine puffs per 3-minute smoking period. The flask contained 1.0 ml of oral fluid-cell harvest. For endogenous activity controls, 2.25 ml of glucose-free dextran solution mixture plus Krebs-Ringer bicarbonate buffer (DSM + KRB), *p*H 7.4, was added; for glucose-dependent activities, 2.00 ml of DSM + KRB and 10 mg of glucose in 0.25 ml of DSM + KRB were added. Temperature, 37°C; atmosphere, 95 percent N_2 plus 5 percent CO_2; period of gassing, 3 minutes.

Oral fluid-cell harvests	Anaerobic glucolysis (CO_2 evolution) (μl)	
	1 hour	2 hours
0–45 minute control	47	113
45–90 minute sample after smoking	23	51
90–135 minute recovery control	38	95

The observations that we have reported here tempt us to speculate upon their implications. Is it possible that chronic exposure to toxic substances of the gas-vapor phase may permanently alter the nature of the inflammatory response? Only long-term experiments can answer this question definitely.

Heretofore, the painting of mouse skins with cigarette smoke condensates has been the most widely used bioassay system in efforts to define the effects that might be deleterious to human smokers. This type of study has given rise to the currently prevalent concept that potential carcinogenic effects are concentrated in the particulate matter or "tars" of cigarette smoke. Our studies suggest an effect of gas-vapor phase constituents on the respiratory, and therefore energy, metabolism of one type of human cell. If one adopts the Warburg-Burk theory (*18*) and postulates that cancer is an ultimate result of impaired cellular respiration, he can speculate that long-term chronic or repetitive exposure of other types of cells in the respiratory system to such gas-vapor phase constituents (or the retained residium thereof) might contribute to production of a malignant transformation. Extensive further studies, especially of a long-term nature, of course, will be required to test any such concept. Nevertheless, undue reliance upon the single model of mouse skin tests with condensates, omitting the gas-vapor phase, is patently unwise.

Of related interest are the recent findings of Leuchtenberger, Schumacher, and Haldiman (*19*), who reported that acrolein has damaging effects involving a sequence of inhibition of RNA synthesis, loss of RNA, pycnosis, and cell destruction (1 to 24 hours after exposure of mouse kidney and slime mold cell cultures to the gas phase of smoke from cigarettes without filters). They found that the gas phase of tobacco smoke, passed through activated-charcoal filters, did not produce alterations of such cell cultures. In contrast, we have found, when using the human oral open test system, that the retained residuum after one activated charcoal cigarette is smoked seriously impairs the function of the peripheral leukocyte. We postulate that this discrepancy indicates an especially high order of sensitivity of the human oral polymorphonuclear leukocyte. More recently Burk, Howard, and Tiggelbeck (*20*) reported direct in vitro inhibitions of the Pasteur effect of cancer and normal cells by 5- to 10-ml volumes of whole tobacco smoke. In their hands, these metabolic effects were considerably eliminated from smoke that had been passed through a Cambridge filter. They believe that the Pasteur effect may provide a fundamental, rapid, and delicate test for assaying relative toxicities of various smokes.

It becomes clear that a number of different bioassay schemes (including ciliary activity depression) have already been developed which appear to measure "presumably undesirable" effects of smoke. Many others may become available. It is possible that the integration of the several systems with long-term animal and human studies may be essential for the development of a "safer" cigarette (*12*).

The study of the chemical composition of the retained residuum as it exists within humans after smoking and the human *in situ* criteria (analogous to the oral cavity open test system employed here) are the most direct routes available toward improved insight in the search for toxicity effects induced by tobacco smoke in vivo.

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Hemoglobins A and A_2 in New World Primates: Comparative Variation and Its Evolutionary Implications

Abstract. *Hemoglobin A_2 ($\alpha_2\delta_2$) in New World primates represents about 1/160 to 1/16 of total hemoglobin and, by virtue of this low proportion, is presumed to be functionally unimportant. Nonetheless, A_2 exhibits genetic polymorphism by electrophoresis in three out of five genera, whereas the major component, hemoglobin A ($\alpha_2\beta_2$), is electrophoretically invariant. Moreover, in four genera, including man, the evolutionary accumulation of mutations has been greater in δ than in β. Such findings suggest that both polymorphism and evolutionary changes can accrue to an effectively functionless and thus selectively nearly neutral gene.*

Some investigators have maintained that demonstrable genetic polymorphism in man (*1*) and *Drosophila* (*2*) is too extensive (*2*) and mutations accumulate too rapidly (*3*) to be easily accounted for by natural selection alone. Consequently it has been supposed that most mutations have little adaptive importance, that is, they are nearly neutral (*3*, *4*), and that much of evolution has proceeded through such mechanisms as genetic admixture and drift. Darwinism is not without defenders (*5*) who argue that the way in which selection operates may have been misjudged and that the rate of adaptive evolution may considerably exceed Haldane's earlier estimate (*6*). Others suggest that natural selection can, in fact, support moderately extensive genetic polymorphism (*7*). Prakash, Lewontin, and Hubby (*8*) have found essential constancy of gene frequencies for a large variety of randomly selected polymorphic proteins in several well-separated, natural populations of *Drosophila*. Such similarity of gene frequencies in different populations is most easily explicable by selection. On the other hand, a large body of molecular evidence analyzed by King and Jukes (*9*) indirectly suggests that most changes in proteins stem from neutral mutations and represent non-Darwinian evolution rather than natural selection. In this connection, direct evidence from the study of variation in nearly functionless genes is lacking, since no very good objects of study have been available. In this report we describe contemporary and evolutionary variation in a *pair* of similar proteins, the adult hemoglobins A and A_2, for which there is substantial physiologic basis for supposing that one member of the pair (A_2) has little or no functional significance and is thus nearly or entirely neutral in adaptive importance.

The distinctive chains of hemoglobins A and A_2 in man presumably arose through gene duplication (*10*). Hemoglobin A_2 appears only in man, apes, and New World primates (*11*). It has not been detected in Old World monkeys. In man, A_2 usually forms less than 3 percent of total hemoglobin while in New World primates it ranges between 0.6 to 6.0 percent (*12*) (Fig. 1 legend). We endeavor to show from comparisons of peptide compositions that the distinctive gene for A_2 arose in an ancestor common to man and New World primates. The low proportion of A_2 in all species suggests that it has not been a major component at any time since the evolutionary divergence of these species. Moreover, there is no evidence that the intracellular proportions of A and A_2 differ substantially from the proportions found in whole blood. The ratio of A to A_2 in man remains approximately constant in postnatal life (*13*), and the distribution of A_2 is essentially homogeneous in erythrocytes of adults (*14*). With these facts as background, we presume (i) that hemoglobin A_2 has little or no functional importance disproportionate to its concentration, (ii) that the functions of adult hemoglobin are principally or entirely served by the major component, hemoglobin A, and therefore, (iii) that A_2 is essentially functionless and thus the δ gene characteristic of A_2 ($\alpha_2\delta_2$) is either neutral in the face of natural selection or, at least, much more nearly so than the β gene characteristic of A ($\alpha_2\beta_2$). If these presumptions are correct, then in man, for example, the present-day adaptive importance of δ should be 3/97, that is, $\sim 1/30$ that of β; thus adaptive variation of δ might be expected to be substantially less than that of β.

In view of such presumptions and in light of the findings of Prakash *et al.* (*8*) that polymorphisms are probably maintained by selection, we were somewhat surprised to find considerable heterogeneity of A_2 in a variety of New World primates (Fig. 1). Three minor hemoglobin types—A_2, W_2, and Y_2—appeared in 106 spider monkeys (*Ateles*). Hemoglobin W_2 occurred only in *Ateles geoffroyi*; the gene frequency in 52 animals was 0.35. Hemoglobin Y_2, although present in three *A. geoffroyi*, was more common in *A. fusiceps* where δY_2 gene frequency in 39 animals was 0.17. Both W_2 and Y_2 appeared in animals from several sources, and thus the common occurrence of these variants is not simply attributable to sampling of closely related individuals. The nature of Y_2 is pertinent; it represents a change from