3. W. W. Douglas, *Br. J. Pharmacol.* **34**, 451 (1968); B. Katz, *The Release of Neurotransmitter Substances* (Thomas, Springfield, Ill., 1969), pp. 1–59; P. F. Baker, *Recent Adv. Physiol.* **9**, 51 (1974); R. P. Rubin, *Calcium and the Secretory Process* (Plenum, New York, 1974), pp. 1–189.
4. G. L. Zink, *Am. J. Pharm.* **146**, 101 (1974); K. F. Austen, R. A. Lewis, D. J. Stechschulte, S. I. Wasserman, R. W. Leid, Jr., E. J. Goetgl, in *Progress in Immunology*, L. Brent and J. Holborow, Eds. (North-Holland, Amsterdam, 1974), p. 61; M. Kaliner and K. F. Austen, *Annu. Rev. Pharmacol.* **15**, 177 (1975); S. I. Wasserman, *Scand. J. Respir. Dis. Suppl.* **98**, 28 (1977); J. Padawer, in *Immediate Hypersensitivity: Modern Concepts and Developments*, M. K. Bach, Ed. (Dekker, New York, 1978), p. 301.
5. T. S. C. Orr, *Br. J. Dis. Chest* **67**, 87 (1973); C. E. Reed, *Med. Clin. North Am.* **58**, 55 (1974); K. F. Austen and R. P. Orange, *Am. Rev. Respir. Dis.* **112**, 423 (1975); N. A. M. Paterson, R. W. Leid, Jr., J. W. Said, S. I. Wasserman, K. F. Austen, in *Lung Cells in Disease*, A. Bouhuys, Ed. (Elsevier, Amsterdam, 1976), p. 223; R. A. Lewis and K. F. Austen, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 2676 (1977); L. M. Lichtenstein and K. F. Austen, *Asthma: Physiology, Immunopharmacology and Treatment* (Academic Press, New York, 1977), pp. 1–141.
6. J. S. G. Cox, in *Pharmacological and Biochemical Properties of Drug Substances*, M. E. Goldberg, Ed. (American Pharmaceutical Association, Washington, D.C., 1977), p. 277; R. G. Coffey and E. Middleton, in *Immunopharmacology*, J. W. Hadden, R. G. Coffey, F. Spreafico, Eds. (Plenum, New York, 1977), p. 203; A. F. Wilson and J. J. McPhillips, *Annu. Rev. Pharmacol. Toxicol.* **18**, 541 (1978); H. Johnson, in *Immediate Hypersensitivity: Modern Concepts and Developments*, M. K. Bach, Ed. (Dekker, New York, 1978), p. 533.
7. C. J. Falliers, *Pediatr. Clin. North Am.* **22**, 141 (1975); E. Middleton, Jr., *ibid.*, p. 111; D. C. Webb-Johnson and J. L. Andrews, *N. Engl. J. Med.* **297**, 476 (1977); *ibid.*, p. 758; M. Weinberger and L. Hendeles, *Postgrad. Med.* **61**, 85 (1977); *ibid.*, p. 958.
8. Several studies have found that 48/80-induced histamine secretion from rat peritoneal mast cells is inhibited by cromolyn in micromolar concentrations (median effective concentration, about 1 to 5 μM) similar to those reported (*16*) to inhibit histamine secretion induced by antigen from the same cells [H. G. Johnson and C. A. Van Hout, *Int. Arch. Allergy Appl. Immunol.* **50**, 446 (1976); H. G. Johnson, in *Immediate Hypersensitivity: Modern Concepts and Developments*, M. K. Bach, Ed. (Dekker, New York, 1978), p. 553; H. G. Johnson, C. A. Van Hout, J. B. Wright, *Int. Arch. Allergy Appl. Immunol.* **56**, 416 (1978)]. Our findings, depicted in Fig. 1, are in accord with this. Other studies have found that cromolyn is slightly less effective [see, for example, M. Chasin, C. Scott, C. Shaw, F. Persico, *ibid.* **58**, 1 (1979)] or much less effective [L. G. Garland and J. L. Mongar, *Br. J. Pharmacol.* **50**, 137 (1974); G. W. Read, M. Knoohuizen, A. Goth, *Eur. J. Pharmacol.* **42**, 171 (1977)] against 48/80. The variation in results may reflect, in part at least, differences in the intensity of the responses elicited by 48/80. It was noted by T. S. C. Orr, D. E. Hall, J. M. Gwilliam, and J. S. G. Cox [*Life Sci.* **10**, 805 (1971)] that the inhibitory effect of cromolyn against 48/80 was lost when the dose of 48/80 was increased to elicit large secretory responses. Additional causes of variation may be the use of purified or unpurified mast cells and also the peculiar bell-shaped dose-response effect of cromolyn in which inhibition tends to diminish as the cromolyn concentration is increased above a certain value (see H. G. Johnson, cited above).
9. J. C. Foreman and L. G. Garland, *Br. Med. J.* **1**, 820 (1976); J. C. Foreman, M. B. Hallett, J. L. Mongar, *Br. J. Pharmacol.* **59**, 473P (1977).
10. W. Sieghart, T. C. Theoharides, S. L. Alper, W. W. Douglas, P. Greengard, *Nature (London)* **275**, 329 (1978).
11. T. C. Theoharides and W. W. Douglas, *Endocrinology* **102**, 1637 (1978).
12. D. C. Morrison, J. F. Roser, P. M. Henson, C. C. Cochrane, *J. Immunol.* **112**, 573 (1974); R. H. Hino, C. K. H. Lau, G. W. Read, *J. Pharmacol. Exp. Ther.* **200**, 658 (1977).
13. P. Röhlich, P. Anderson, B. Uvnäs, *J. Cell Biol.* **51**, 465 (1971); M. Kagayama and W. W. Douglas, *ibid.* **62**, 519 (1974).
14. W. W. Douglas and Y. Ueda, *J. Physiol. (London)* **234**, 97P (1973); E. Woldemussie and N. C. Moran, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 603 (1978); J. H. Baxter and R. Adamik, *Biochem. Pharmacol.* **27**, 497 (1978).
15. L. G. Garland, *Br. J. Pharmacol.* **49**, 128 (1973); E. J. Kusner, B. Dubnick, D. J. Herzig, *J. Pharmacol. Exp. Ther.* **184**, 41 (1973).
16. Cromolyn did not block the 48/80-induced phosphorylation of the protein bands of molecular weights 68,000, 59,000 and 42,000.
17. P. V. Sulakhe and P. J. St. Louis, *Gen. Pharmacol.* **7**, 313 (1976); A. Wollenberger and H. Will, *Life Sci.* **22**, 1159 (1978); A. M. Katz, *Adv. Cyclic Nucleotide Res.* **11**, 303 (1979).
18. R. Kaser-Glanzmann, M. Jakabova, J. N. George, E. F. Luscher, *Biochim. Biophys. Acta* **466**, 429 (1977).
19. B. K. Krueger, J. Forn, P. Greengard, *J. Biol. Chem.* **252**, 2773 (1977).
20. It was important to keep the purified mast cells (which had been washed free of bovine serum albumin) on ice and to use them as quickly as possible because such cells rapidly lose their responsiveness to 48/80 at room temperature [K. Saeki, *Jpn. J. Pharmacol.* **14**, 375 (1964)].
21. L. T. Kremzner and I. B. Wilson, *Biochim. Biophys. Acta* **50**, 364 (1961).
22. Supported by PHS grants DA-01627, MH-17387, and NS-08440 to P.G. and NS-09137 to W.W.D.; W.S. was a Max Kade Fellow. We thank G. B. Eagleton for the gift of cromolyn.
* Present address: Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn. 06510.
† Present address: Psychiatrische Universitäts Klinik, Lazarettgasse XIV, 1097 Wien, Austria.

11 May 1979; revised 6 August 1979

Intraclonal Variation in Proliferative Potential of Human Diploid Fibroblasts: Stochastic Mechanism for Cellular Aging

Abstract. *At several points during the growth of a clone of human embryonic lung fibroblasts in vitro, 100 to 200 cells were removed at random and the proliferative potential of each cell was determined. At each sample point, a wide variation in remaining population doubling ability was observed among the individual cells and the distributions of doubling potentials were distinctly bimodal. Furthermore, the two cells arising from a single mitosis differed in their ability to proliferate by as many as eight population doublings (256-fold in the number of cells produced). The results suggest that a stochastic process is responsible for determining the limited proliferative potential of human embryonic lung fibroblasts.*

Normal human diploid fibroblasts have a limited life-span in vitro (that is, a finite proliferative potential in culture) (*1*). This potential has been shown to be inversely proportional to the age of the tissue donor (*2*) and has been widely studied as a model for cellular aging. Numerous biochemical and morphological changes occur as these cells reach the end of their proliferative potential (*1*). A number of hypotheses have been proposed to explain this phenomenon; however, direct biochemical analysis of cultures has not yet identified the mechanisms responsible for limiting the proliferative potential of normal human cells.

Many investigators (*3–6*) have reported heterogeneity in morphology, interdivision time, and ability for clonal growth among the individual cells of mass cultures of human diploid fibroblasts. At all stages in the life-span in vitro of human diploid fibroblast cultures (WI-38 cells), wide variation in the proliferative potential of isolated clones was observed (*7*). From these experiments, it was not possible to determine whether variability continues to develop during repeated doubling of the culture in vitro or whether the variation is due to heterogeneity among the individual cells in the tissue of origin. Alternatively, the variation might develop during the process of initiating the cells in culture. A more detailed investigation of this variation would provide some insight into the mechanisms determining proliferative potential. In addition, this information would provide a powerful data base against which to test hypotheses. In the experiments reported here, we found that heterogeneity in proliferative potential appears rapidly within a single clone of human diploid fibroblasts. We also found that the proliferative potentials of the two cells from a single mitotic event can differ by as many as eight population doublings (PD's).

To determine the intraclonal variation in proliferative potential, the distributions of doubling potentials were determined for three sets of subclones isolated from a single clone that was initiated from a culture of human embryonic lung cells (Flow 2000) (*8*) in vitro at PD 23. Two hundred cells were isolated at random from the parent clone at 16, 26, and 36 PD's after its initiation as a single cell (see legend to Fig. 1). Each subclone was observed until the limit of its replicative ability was reached, and frequency distributions of proliferative potential (the number of PD's achieved after isolation) were constructed for each set of subclones (Fig. 1). Cultures were considered to be at the end of their proliferative potential when they failed to double in cell number for 2 weeks. At PD 16, one of the subclones was used to initiate a second set of subclones, and the frequency distribution of their proliferative potential was determined (Fig. 1, inset).

Several characteristics of the distributions are noteworthy: (i) the parent clone was more homogeneous with respect to

0036-8075/80/0104-0082$00.50/0

the proliferative potential of its individual cells than are mass cultures of the same cell type (*7*); (ii) within only 16 doublings after starting from a single cell, the resulting clone contained cells ranging in doubling potential from 0 to 33 PD's (Fig. 1a); and (iii) the distributions are distinctly bimodal except for the one obtained nearest the end of proliferation of the parent clone (Fig. 1c), where the two modes seem to have merged into a single mode.

In view of the rapid development of intraclonal variability in doubling potential, we considered it important to determine the degree of difference in doubling potential between the two cells arising from a single mitotic event. In Fig. 2, the results of these experiments are presented in terms of the normalized frequency distribution of the proliferative potentials of one of the cells of a mitotic pair whose other cell was able to undergo the indicated number of doublings (each histogram combines the results from several mitotic pairs). It is apparent that the proliferative potential of the two cells from a single mitotic event can differ by as many as eight PD's.

Our results agree with those of studies (*3*-*6*) in which heterogeneity in interdivision time and in growth rate within individual clones is shown, support the observation (*9*) that a large fraction of the cells in a normal culture have a very low proliferation potential and therefore cannot form large clones, and support the finding (*10*) that a bimodality of colony size results from a 1- to 2-week incubation of repeatedly subcloned human diploid cells and HeLa cells. In addition, we have demonstrated that there is marked variation in the remaining proliferative potential among the cells within single clones of human embryonic lung fibroblasts.

The rapid development of intraclonal variation and the large differences between the products of single mitotic events strongly suggest that a stochastic process determines the doubling potential of human diploid cells. As a clone undergoes doublings in vitro there is a gradual loss in the remaining proliferative potential of its individual cells (the large doubling-potential mode shown in Fig. 1, a to c, decreases), indicating multievent processes. The degree of variability in the doubling potential of the subclones within the large doubling-potential mode is greater than that expected from the variable interdivision time previously observed (*11*). Furthermore, this variability is probably not due to contact inhibition of cells in the interior portion of the original parent clone, since the proportion of cells incorporating ^{3}H-labeled thymidine during a 24-hour exposure was approximately the same for cells in the center and on the periphery of the clone (70 and 80 percent, respectively). The intrinsic nature of the distribution of cells about the large doubling-potential mode is further argued by the high degree of variability between pairs of cells resulting from one mitosis (Fig. 2). In a series of experiments (*12*), we found that under the proper clonal conditions, human embryonic lung fibroblasts had the same PD time, proportion of nondividing cells, and proliferative potential when continuously cultured at clonal densities (one to four cells per square centimeter) and at mass-culture densities (1×10^4 cells per square centimeter). With respect to the data presented in Fig. 1, this evidence indicates that a subpopulation of cells with very low doubling potential exists in the parental population and must be considered when searching for the mechanisms that limit proliferative potential.

We conclude that two kinds of quantitative events occur in this context: one that results in a gradual decrease in proliferative potential and one that results in an abrupt transition from the larger to the smaller (six or fewer) doubling fre-

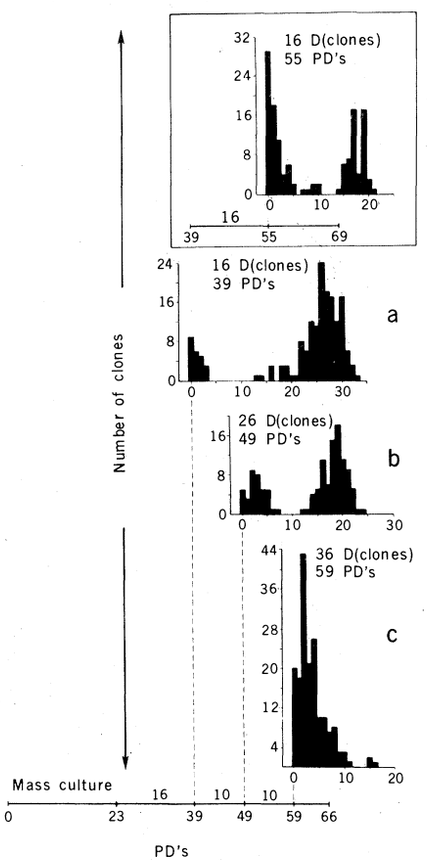

Fig. 1. Intraclonal variation in proliferative potential. Frequency distribution of proliferative potential of subclones isolated from a single clone is shown at (a) 16, (b) 26, and (c) 36 PD's after initiation of the parent clone. The frequency distribution of a secondary subclone is shown in the inset; this set of subclones was isolated from one of the subclones of group (a) 16 PD's after initiation of the primary subclone. The distribution of doubling potential of subclones was determined by the following procedure. Two days after subculture in Eagle's MEM supplemented with 28 m*M* Hepes buffer and 10 percent fetal bovine serum, a single cell suspension was prepared by trypsinization of the log phase culture. The cells were cultured at low density (1000 to 5000 cells per 60-mm dish) in medium MCDB 102 (*16*) supplemented with 10 percent fetal bovine serum in tissue culture dishes containing small cover-glass fragments (*17*). Clones were then isolated and grown as described in (*7*).

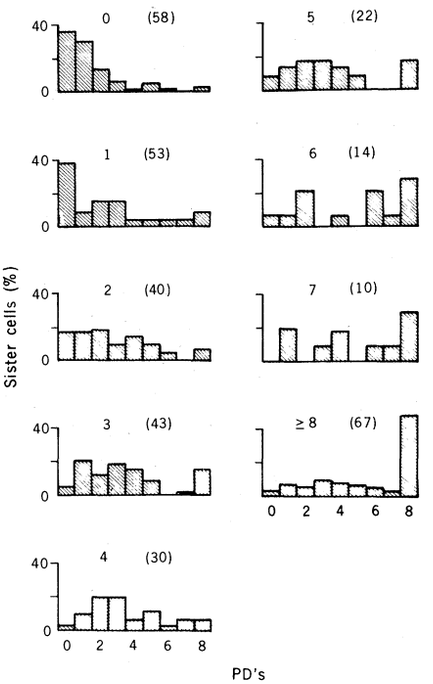

Fig. 2. Variation in proliferative potential between cells arising from single mitotic events. The results are presented as the normalized frequency distributions of doubling potentials of cells whose sister cells were able to undergo the indicated number of doublings. The number of mitotic pairs analyzed are given in parentheses. Cells were cultured in 60-mm dishes containing 5 ml of MCDB 102 or Eagle's MEM in 10 percent fetal bovine serum. There were 50 to 100 cells per dish. The day after seeding, single cells were isolated by cutting narrow grooves (in the form of a square) around them. (Control experiments showed that in 98 of 100 cases, cells were unable to cross the groove cut into the plastic dishes.) During the next 24 hours, the squares were intermittently examined to see if any of the cells within the squares had divided. If a cell had divided, a groove was cut between the two sister cells. After 2 weeks, the dishes were stained in crystal violet (0.5 percent) and the number of doublings achieved by each sister cell was determined by counting with a dissecting microscope. A maximum of 256 cells from either daughter cell of a mitotic pair could be counted with precision, therefore all cells giving rise to 256 or more cells are considered as a single category of ≥ 256 cells.

quencies; the rate of this transition appears to increase as the proliferative potential of the clone decreases (Fig. 1a and inset).

The information given in this report should be useful for testing hypotheses that seek to explain the limited doubling potential of human diploid cells. It is clear that neither a precise counting mechanism (*13*) nor the commitment theory of aging (*14*) are compatible in their current form with our findings. However, if one considers the involvement of stochastic mechanisms in stem cell differentiation (*15*), our data may be seen as compatible with a process of genetically controlled terminal differentiation, such as has been proposed by Martin *et al.* (*4*) and Bell *et al.* (*5*). The gradual decrease in proliferative potential would also be compatible with a continuous buildup of damage or errors, a process that has been theorized. However, the wide variability in doubling potentials, especially in mitotic pairs, suggests an unequal partitioning of damage or errors at division. The transition of the proliferative potential subset from large to small suggests the occurrence of a more serious species of damage or the crossing of a threshold level of accumulated damage. In terms of somatic mutation theory, the wide variation at each cell division suggests mutation rates (nonlethal but capable of affecting proliferative potential) approaching one per cell division. Clearly, all current hypotheses should be reexamined in the light of our data.

JAMES R. SMITH
RONALD G. WHITNEY
W. Alton Jones Cell Science Center, Lake Placid, New York 12946

References and Notes

1. L. Hayflick, in *Handbook of the Biology of Aging*, C. E. Finch and L. Hayflick, Eds. (Van Nostrand Reinhold, New York, 1977), p. 159.
2. G. M. Martin, C. A. Sprague, C. J. Epstein, *Lab. Invest.* **23**, 86 (1970); Y. LeGuilly, M. Simon, P. Lenoir, M. Bouree, *Gerontologia* **19**, 303 (1973); E. L. Schneider and Y. Mitsui, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3584 (1976).
3. P. M. Absher, R. G. Absher, W. K. Barnes, *Exp. Cell Res.* **88**, 94 (1974).
4. G. M. Martin *et al.*, *Am. J. Pathol.* **74**, 137 (1974).
5. E. Bell, L. F. Marek, D. S. Levinstone, C. Merrill, S. Sher, I. T. Young, M. Eden, *Science* **202**, 1158 (1978).
6. G. S. Merz and J. D. Ross, *J. Cell. Physiol.* **74**, 219 (1969); V. J. Cristofalo and B. B. Sharf, *Exp. Cell Res.* **76**, 419 (1973).
7. J. R. Smith and L. Hayflick, *J. Cell Biol.* **62**, 48 (1974).
8. Flow 2000 cells, human embryonic lung fibroblast-like cells, were obtained from Flow Laboratories at population-doubling level 18.
9. J. R. Smith, O. M. Pereira-Smith, E. L. Schneider, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1353 (1978); J. R. Smith, O. Pereira-Smith, P. I. Good, *Mech. Ageing Dev.* **6**, 283 (1977).
10. G. M. Martin, C. A. Sprague, T. H. Norwood, W. R. Pendergrass, *Am. J. Pathol.* **74**, 137 (1974); A. O. Martinez, T. H. Norwood, J. W. Prothero, G. M. Martin, *In Vitro* **14**, 996 (1978).
11. Based on the data reported by Absher *et al.* (*3*) for a clone with the same average interdivision time as the one we studied (doubling every 20 hours), we calculated that variable interdivision time alone would result in a doubling-potential distribution with a standard deviation of 0.80 PD. This is considerably smaller than the standard deviation of 3.7 PD we observed.
12. J. R. Smith and K. I. Braunschweiger, *J. Cell. Physiol.* **98**, 597 (1979).
13. P. I. Good and J. R. Smith, *Biophys. J.* **14**, 811 (1974).
14. T. B. L. Kirkwood and R. Holliday, *J. Theor. Biol.* **53**, 481 (1975).
15. J. E. Till, E. A. McCulloch, L. Siminovitch, *Proc. Natl. Acad. Sci. U.S.A.* **51**, 29 (1964).
16. R. G. Ham, S. L. Hammond, L. L. Miller, *In Vitro* **13**, 1 (1977).
17. G. M. Martin and A. Taun, *Proc. Soc. Exp. Biol. Med.* **123**, 138 (1966).
18. Supported by NIH grant R01-AG00338 and the W. Alton Jones Foundation.

17 July 1979; revised 10 October 1979

Entry of Opioid Peptides into the Central Nervous System

Abstract. *Cerebrovascular permeability of four modified opioid peptides—[D-Ala²]methionine enkephalin amide, β-[D-Ala⁶², ¹⁴C-Homoarg⁶⁹]lipotropin 61-69, α-[D-Ala², ¹⁴C-Homoarg⁹]endorphin, and β-[D-Ala², ¹⁴C-Homoarg]endorphin—ranged from 1.4 to 3.9 × 10⁻⁶ centimeters per second in brain regions of the conscious rat. These significant permeabilities should allow the peptides to fill the extracellular brain space with a half time of 3 to 11 minutes, as a result of a step increase in plasma concentration of unbound peptide.*

Some peptides that are derived from the pituitary hormone β-lipotropin are reported to exert central effects when injected systemically, but it is not clear why they do so as they often are reported to be slightly if at all permeable at the blood-brain barrier (*1-4*). An insignificant permeability would appear consistent with the view that the blood-brain barrier, which is composed of cerebrovascular endothelial cells that are connected by tight junctions (zonulae occludentes), is essentially impermeable to water-soluble nonelectrolytes, electrolytes, peptides, and proteins (*5*, *6*). Because of their limited cerebrovascular permeability (*2*), it has been suggested that circulating peptides enter the brain via the choroid plexus and cerebrospinal fluid or, if produced in the pituitary gland, through routes such as the pituitary portal system (*1*).

In contrast, some investigators have reported that peptides are significantly permeable at the blood-brain barrier (*7*). These findings, and the observed positive central effects to some systemically administered peptides (*3*), suggested to us that cerebrovascular permeability of peptides should be reconsidered. Moreover, the method (brain uptake index) most used to show that peptides do not cross the blood-brain barrier, is not sensitive enough to measure uptake of substances whose permeability coefficients are less than 10^{-6} cm sec^{-1} (*2*, *6*, *8*).

We therefore employed a more sensitive method (*9*) to measure cerebrovascular permeability of four radioactive synthetic opioid peptides that are analogs of natural peptides derived from β-lipotropin (*4*, *10*). These synthetic peptides shared a common amino acid terminal sequence, Tyr-D-Ala-Gly-Phe-Met- (*11*), in which the D-alanine residue was placed in position 2 to increase resistance to degradation without significantly reducing biological activity (*4*). They were: [D-Ala²]methionine enkephalin amide; β-[D-Ala⁶², ¹⁴C-Homoarg⁶⁹]lipotropin 61-69; α-[D-Ala², ¹⁴C-Homoarg⁹]-endorphin; and β-[D-Ala², ¹⁴C-Homoarg]-endorphin. They corresponded, respectively, to the following natural peptides: Met enkephalin, β-lipotropin 61-69, α-endorphin, and β-endorphin (*10*).

Conscious and partially restrained rats (adult males) with indwelling femoral artery and vein catheters were injected intravenously with 2 to 10 μCi of a radioactive opioid peptide; samples of arterial blood were removed periodically and centrifuged (*9*). Animals were decapitated 3 minutes after injection of [D-Ala²]methionine enkephalin amide; 3 or 10 minutes after β-[D-Ala⁶², ¹⁴C-Homoarg⁶⁹]lipotropin 61-69; 4, 8, 20, or 30 minutes after α-[D-Ala², ¹⁴C-Homoarg⁹]endorphin; and 5 or 10 minutes after β-[D-Ala², ¹⁴C-Homoarg]endorphin. The brain was removed and dissected into 13 regions, which were weighed. Radioactivity was determined by scintillation counting in samples of brain, arterial plasma, and whole blood. Brain parenchymal tracer concentration (C_{brain}, dpm/g) was calculated by subtracting intravascular radioactivity from net measured regional radioactivity. Intravascular radioactivity was obtained from the product of whole blood concentration (dpm/ml) × regional blood volume (ml/g), where regional volumes ranged from 0.015 to 0.035 ml per gram of brain (*9*).

Tracer purity was confirmed by chromatography of the injected material and of the plasma up to the time that the animal was decapitated. The fraction *r* of tracer not bound to blood proteins was determined by mixing tracer with rat serum and measuring radioactivity in serum and serum ultrafiltrate. The octa-

0036-8075/80/0104-0084$00.50/0