Primary Structure of Mouse, Rat, and Guinea Pig Cytochrome c[†]

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ABSTRACT: For immunochemical and evolutionary reasons, we determined the primary structure of cytochrome c from two strains of laboratory mice. Thioacetylthioethane and thioacetylthioglycolic acid were used in addition to conventional reagents for sequence determinations. The sequence was found to be identical with that of the rabbit except for residues 44 and 89 and consistent with the peptide compositional data reported by Hennig (Hennig, B. (1975), *Eur. J. Biochem. 55,* 167–183). The rat cytochrome c chymotryptic peptides were identical with those of the mouse in amino acid composition and amino-terminal residues. Further, peptide maps of cytochromes c of the guinea pig and two strains of rat indicate that all these animals have the same cytochrome c as the laboratory mouse. It is concluded that rodent cytochromes c are evolutionarily conservative and that there is no evidence for a generation-time effect in cytochrome c evolution.

To understand the mechanism of evolution, it is essential to study the rates at which evolutionary change has taken place in the sequences of macromolecules. The claim that generation-time affects the rate of molecular evolution (Laird et al., 1969; Kohne, 1970), although contested (Sarich, 1972; Sarich and Wilson, 1973), has been widely accepted (Hood et al., 1975; Benveniste and Todaro, 1975). The evidence for this claim comes mainly from studies of rates of macromolecular sequence change in higher primates, which have long generations, and rodents, whose generations are short. The macromolecules studied explicitly in this connection include nonrepetitive DNA, with an annealing method (Laird et al., 1969; Kohne, 1970), serum albumin with an immunological method (Sarich, 1972; Sarich and Wilson, 1973), and lysozyme by amino acid sequencing (White et al., 1977). Since sequence information is available for the cytochromes c of some primates (Dayhoff, 1972), we decided to obtain similar information from several species of rodents (mouse, rat, and guinea pig). This enabled us to compare the rates of cytochrome c evolution in rodents and primates. In contrast to the prediction of the generation-time hypothesis, rodent cytochromes c have changed less from the ancestral mammalian state than have those of primates.

There was also an immunochemical reason for undertaking this sequence study. Because the series of cytochromes c of known primary structure are excellent reagents for the study at the molecular level of the immunological response to globular proteins (Reichlin et al., 1966; Margoliash et al., 1967; Margoliash et al., 1970; Nisonoff et al., 1970; Reichlin et al., 1970), it became important to determine the amino acid sequence of mouse cytochrome c. Indeed, the mouse is an experimental animal commonly employed for the production of antibodies and for the investigation of other immune phenomena. The amino acid sequence of mouse cytochrome c was found to be identical with that of the rabbit except for residues 44 and 89. After this work was completed, a study by Hennig (1975) showed that mouse cytochrome c had that structure. However, since only the amino acid compositions of the tryptic peptides were determined, and for immunological experiments it is essential to be certain of the amino acid sequence, it was considered useful to report the present complete determination of the structure of mouse cytochrome c.

Further, rat and guinea pig cytochromes c are probably identical with mouse cytochrome c. This was evident from compositional studies on the intact proteins as well as from compositional analysis, amino-terminal analysis and chromatographic and electrophoretic data on peptide fragments. A preliminary account of some of these findings has appeared (White et al., 1975).

Experimental Procedure

Thioacetylthioethane was synthesized by the method of Marvel et al. (1955). Thioacetylthioglycolic acid was synthesized by combining the methods of Marvel et al. (1955) and Jensen and Pederson (1961).

Cytochrome c Preparations. The cytochromes c were purified by the procedure of Margoliash and Walasek (1967). The cytochromes c were obtained from the skinned eviscerated carcasses from which the testes had been removed.

Purified rat and mouse monomeric cytochrome c migrated as a single band toward the cathode during electrophoresis at pH 5.5 in starch gels under the conditions described by Prager and Wilson (1971).

Amino acid compositions of proteins and peptides were determined with a Durrum D-500 or a Beckman 121 automatic amino acid analyzer, following hydrolysis in 6 N HCl at 110 °C in vacuo for 20 to 72 h. Tryptophan was determined by toluenesulfonic acid hydrolysis (Liu and Chang, 1971). Hydrolysates containing homoserine were treated according to Ambler (1965) and Corradin and Harbury (1970) to obtain good quantitation of this amino acid.

Cyanogen bromide cleavage of mouse and rat cytochrome c was done either by the methods of Corradin and Harbury (1970) or Gross (1967). The resulting peptides were separated either by column chromatography on Sephadex G-50 or G-25.

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Enzymic Digestions. Tryptic and chymotryptic digestions of BALB/c mouse or Sprague-Dawley rat cytochrome *c* or peptides from these proteins were carried out in 0.1 M NH_4HCO_3 (pH 8.0) at an enzyme concentration of 0.1 mg/mL and an enzyme substrate ratio of 1/50 for 8 h at 37 °C. The chymotryptic digestions of the cytochromes *c* were preceded by the ethanol denaturation treatment of Margoliash et al. (1962) without the 5 M ammonium acetate precipitation and dialysis.

Tryptic digestion of Webster-Swiss mouse cytochrome *c* was performed as previously described (Nolan and Margoliash, 1966).

Thermolysin digestions of heme peptides were performed in a solution containing 0.1 M NH_4HCO_3 and 0.2 mM $CaCl_2$ (pH 8.0) for 18 h at 37 °C. The enzyme concentration was 0.05 mg/mL and the enzyme substrate weight ratio was 1/62.

Sixty nanomoles of the cyanogen bromide fragment representing residues 81–104 of mouse cytochrome *c* was digested with carboxypeptidase A (enzyme concentration, 0.6 mg/mL; peptide concentration, 0.6 nmol/μL) in a buffer containing sodium acetate (0.028 M) and sodium barbitol (0.028 M) at pH 6.0.

Partial acid hydrolysis was performed on amino-terminal peptides of mouse cytochrome *c*. The amino-terminal chymotryptic peptide (230 nmol) of BALB/c mouse cytochrome *c* was hydrolyzed in 0.5 mL of 0.03 N HCl–40% acetic acid for 18 h at 107 °C under a vacuum. The amino-terminal tryptic peptide (5 μmol) of Webster-Swiss mouse cytochrome *c* was hydrolyzed in 5 mL of 0.1 M acetic acid for 7 h at 110 °C under a vacuum.

Peptide Purification by Column Chromatography. Peptides resulting from a chymotryptic digestion of the BALB/c mouse cytochrome *c* cyanogen bromide fragment representing residues 1–65 were chromatographed on Aminex A-5 (Bio-Rad Laboratories) using the systems described by Schroeder (1967) and Jones (1970). Sephadex G-15 column chromatography (in 0.2 M pyridine–acetate buffer pH 6.0) was utilized in some cases to further purify these peptides.

Tryptic peptides of Webster-Swiss mouse cytochrome *c* were prepared as previously described (Nolan and Margoliash, 1966).

The Chymotryptic Heme Peptide. Since the yield of the heme peptide from Aminex A-5 was poor, more of it was purified from mouse (BALB/c) and rat (Sprague-Dawley) cytochrome *c* by cochromatography of chymotryptic digests of these molecules with bovine serum albumin on Sephadex G-75. Due to the weak interaction between the heme peptide and the albumin, the heme peptide eluted behind the serum albumin and in front of the majority of the cytochrome *c* chymotryptic peptides.

The purification of the heme peptide was completed by chromatography on a Sephadex G-50 column equilibrated with 2 M formic acid at 4 °C.

Peptide Purification by Paper and Thin-Layer Procedures. Peptides were further purified by using various combinations of the techniques of high voltage paper electrophoresis, paper chromatography, ascending thin-layer chromatography, and low voltage paper electrophoresis.

High voltage paper electrophoresis was carried out as previously described (Margoliash and Smith, 1962) or using the electrophoretic system described by Bennett (1967) with a pH 6.5 pyridine–acetate buffer or a pH 1.9 formic acid buffer. Electrophoresis was carried out at 3000 V and the charge of the peptides was determined by the method of Offord (1966).

Paper chromatography was carried out as previously described (Margoliash and Smith, 1962) or utilizing the 1-butanol–pyridine–acetic acid–H_2O solvent system of Bennett (1967).

Ascending thin-layer chromatography was carried out on cellulose sheets (Eastman Chromagnam sheet 6064 cellulose) with solvent system 1-butanol–pyridine–propionic acid–H_2O (90:60:18:72).

Low voltage paper electrophoresis was performed at 300 V with either 2% formic acid–8% acetic acid (pH 2.0) or 0.1 M pyridine–acetate (pH 4.1).

Thin-Layer Peptide Maps. Tryptic and chymotryptic digestions of Webster-Swiss mouse, Wistar rat, and guinea pig cytochrome *c* (1 to 2 mg of cytochrome *c*; 5% by weight enzyme; 100 μL of 0.05 M ammonium bicarbonate; 3 to 6 h at 37 °C or 6 to 9 h at room temperature) were stopped by immersion in boiling water (3 to 4 min), the tubes centrifuged, and the supernatants lyophilized. The digests were dissolved in 20 μL of water, and 1 μL of the solution was placed on 20 × 20 cm Brinkman cellulose thin layer sheets. Then electrophoresis (400 V; pH 6.5, pyridine–acetic acid–water, 200:7:1800, v/v; 60 and 75 min for the tryptic and chymotryptic digests, respectively) and ascending chromatography (1-butanol–pyridine–acetic acid–water, 60:40:12:48, v/v) were carried out. To determine whether digests from the three cytochromes gave identical peptide maps, equal amounts of digests from two proteins were mixed and the resultant maps compared with maps run with the same separate digests.

Peptides were detected on paper or thin-layer sheets by either ninhydrin (Nolan and Margoliash, 1966) or fluorescamine. When fluorescamine was used, the electrophoretograms or chromatograms were first sprayed with 3% pyridine in acetone and then 0.1 mg/mL fluorescamine in acetone. The dry sheet was then viewed under ultraviolet light.

Removal of heme from heme peptides was accomplished either by reaction with mercuric chloride according to Ambler (1963), and the cysteines carboxymethylated (Crestfield et al., 1963), or by performic acid oxidation (Ramshaw et al., 1970).

Edman sequential degradation of peptides was carried out manually as previously described (Jeppson and Sjoquist, 1967; Keresztes-Nagy et al., 1969; Nolan et al., 1971, 1973; Summers et al., 1973) or in combination with the dansyl amino-terminal identification technique (Hartley, 1970).

Solid-Phase Sequencing. The solid-phase sequencing method of Mross and Doolittle (Mross, 1971; Mross and Dolittle, 1971; Prager et al., 1972) and the carboxy-terminal homoserine resin attachment procedure of Horn and Laursen (1973) were used to sequence one 15-residue peptide. The procedure was automated by G. Mross in the laboratory of A. C. Wilson. This solid-phase sequencer was very similar to that of Laursen (1971).

Sequential Degradation with Thioacetylthioethane (TATE).[1] TATE is a derivative of thioacetylthioglycolic acid and was used in combination with the dansyl procedure for the sequential degradation of peptides in solution. The procedure was similar to the Edman degradation except that it did not include a butyl acetate or butyl chloride extraction. It was as follows.

(1) Two hundred microliters of 50% pyridine–1% triethylamine and 20 μL of TATE were added to 60–100 nmol of dry peptide in a 12-mL conical screw-cap centrifuge tube. This was

[1] Abbreviations used: UV, ultraviolet; TATE, thioacetylthioethane.

TABLE I: Amino Acid Compositions of Mouse, Rat, and Guinea Pig Cytochrome *c*.

Amino Acid	Residues/Molecule[a]				
	Mouse Cytochrome *c*		Rat Cytochrome *c*		Guinea Pig Cytochrome *c*
	BALB/c	Webster-Swiss	Sprague-Dawley	Wistar	
Asp	9.23	8.91 (9)	9.34	8.70	8.98
Thr[b]	7.75	7.87 (8)	7.65	7.63	7.98
Ser[b]	0.97	1.33 (1)	1.02	1.44	1.14
Glu	10.53	10.17 (10)	10.33	10.00	9.98
Gly	14.15	13.94 (14)	14.53	13.83	13.78
Ala	7.66	8.22 (8)	8.04	8.23	7.79
½-Cystine	ND	ND (2)	ND	ND	ND
Pro	3.21	ND (3)	3.15	ND	ND
Val	2.95	2.97 (3)	3.00	2.90	2.92
Met	1.97	1.75 (2)	1.86	2.28	1.79
Ile	5.80	6.00 (6)	5.76	6.29	6.10
Leu	5.96	6.00 (6)	6.15	6.00	6.10
Tyr	3.81	3.99 (4)	3.87	3.80	4.30
Phe	3.81	4.00 (4)	3.72	3.81	4.27
Trp	0.99	ND (1)	0.88	ND	ND
His	2.91	2.96 (3)	2.90	2.74	3.05
Lys	18.13	17.88 (18)	17.88	18.05	17.74
Arg	2.10	2.06 (2)	1.82	2.11	2.14

[a] The proteins were hydrolyzed in duplicate or triplicate for 24, 48, and 72 h. The values listed are averages or extrapolated to zero time. The values in parentheses are those obtained from the amino acid sequence of mouse cytochrome *c*. ND, not determined. [b] The threonine and serine peaks tend to overlap sufficiently in most of the amino acid analyses so that, when there is much more threonine than serine, the former is underestimated, while the latter is overestimated. Some serine is also a decomposition product of the thioether bonded cysteines of cytochrome *c* (Margoliash et al., 1962).

more than enough TATE to saturate the solution, and some of the TATE remained undissolved at the bottom of the tube. The tube was then flushed with N_2, the solution mixed, and the reaction mixture incubated for 1 h at 45 °C.

(2) The reaction mixture was then evaporated on high vacuum over NaOH pellets and concentrated H_2SO_4 in a desiccator heated to 60° °C.

(3) An aliquot of 0.7 mL of trifluoroacetic acid was added to the dry thioacetylated peptide flushed with N_2; the tube was then capped and incubated at 45 °C for 45 min. At the end of this time, the trifluoroacetic acid was removed in a desiccator by a water-aspirator vacuum. The desiccator was then put on a high vacuum for about 10 min.

(4) Three hundred microliters of methanol was added to the dry peptide and incubated at room temperature for 5 min. The methanol was then evaporated by a stream of N_2.

(5) One hundred microliters of 50% pyridine was added and the appropriate aliquot taken for dansyl N-terminal analysis. The pyridine solution was then lyophilized over concentrated H_2SO_4.

The thioacetylthioethane degradation, originated by George Mross in the laboratory of A. C. Wilson, was used in combination with the dansyl amino-terminal identification procedure. Since the reagent as well as its hydrolysis products are very volatile, the extraction step of the dansyl-Edman procedure (Hartley, 1970) can be avoided. Thus, the thioacetylthioethane–dansyl procedure was thought to offer a possible advantage in reducing peptide losses in the sequential degradation of easily extractable peptides. Although this study presents no information which relates directly to this possible advantage, it does indicate that for average peptides of about 6 residues the thioacetylthioethane–dansyl procedure is comparable to the dansyl-Edman. The possible realization of this advantage must await further study.

Results

The amino acid compositions of the two mouse, the two rat, and the guinea pig cytochromes *c* are listed in Table I. They are all clearly identical.

Sequence Studies on BALB/c Mouse Cytochrome c. BALB/c mouse cytochrome *c* was cleaved with cyanogen bromide and the three major cleavage products isolated: C_1 (residues 1–65), C_2 (residues 66–80), and C_3 (residues 81–104).

Fragment C_1 was digested with chymotrypsin and the resulting peptides were separated by chromatography on Dowex-50 (Aminex A5). For some peptides further purification was required utilizing the techniques of high voltage paper electrophoresis, ascending thin-layer chromatography, or Sephadex G-15 column chromatography. The peptide which represents residues 1–10 was blocked at the N terminus like the N-terminal peptide of all sequenced vertebrate cytochromes *c*. It was subjected to partial acid hydrolysis and the products were isolated by low voltage electrophoresis. The major cleavage products detected were free glycine, free aspartic acid, and an octapeptide. The blocking group which, by homology with other sequenced cytochromes *c*, was undoubtedly an acetyl group must have been released by the partial acid hydrolysis.

The yield of the chymotryptic heme peptide from the Aminex A5 column was poor and more of it was needed for a complete sequence analysis. This peptide was easily purified by cochromatography of a chymotryptic digest of mouse cytochrome *c* with purified bovine serum albumin on Sephadex G-75. This peptide was then digested by trypsin and thermolysin and the resulting peptides were purified by low voltage electrophoresis.

The C_2 cyanogen bromide fragment (residues 66–80) was

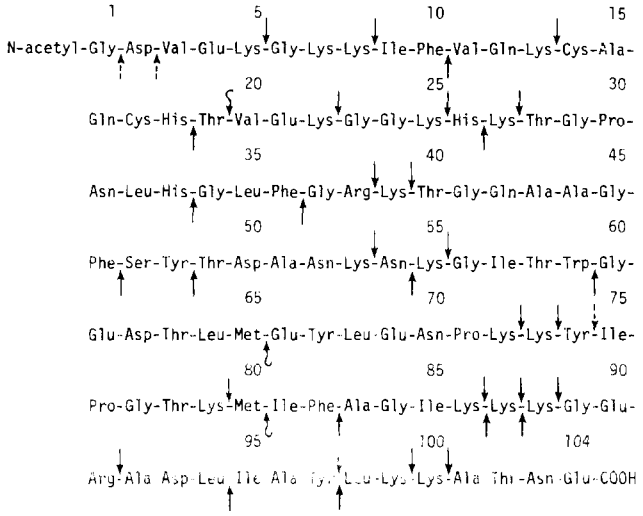

FIGURE 1: Amino acid sequence of mouse cytochrome *c*. The arrows indicate cleavages by trypsin (↓), chymotrypsin (↑), thermolysin (⇓), cyanogen bromide (⇑), and partial acid hydrolysis (↕). Atypical chymotryptic-like cleavages by trypsin are indicated as ⇓.

completely sequenced by solid-phase sequencing techniques. One hundred and six nanomoles of this peptide was successfully coupled to the resin and the average recovery of each amino acid residue per step was 87%. The degradation was 3% out of phase at step 3 and 26% at step 12. The amides and acids in the peptide were identified by examining the phenylthiohydantoin amino acids resulting from a separate manual Edman degradation of the first 5 residues of this peptide.

Cyanogen bromide peptide C_3 (residues 81–104) was digested by both trypsin and chymotrypsin and the resulting peptides were isolated by high voltage paper electrophoresis.

Peptides accounting for the entire primary structure of mouse (Balb/c) cytochrome *c* were sequenced. Except for peptide C_2, peptides were sequenced by the TATE–dansyl or dansyl–Edman procedure. Amides were determined by phenylthiohydantoin amino acid identification, electrophoretic mobility at pH 6.5, or in the case of the last two residues of peptide C_3 carboxypeptidase A digestion.

The positions of the three cyanogen bromide fragments were arranged by comparison with other sequenced cytochromes *c*. The chymotryptic peptides within the cyanogen bromide representing residues 1–65 were placed in the sequence also by homology (Dayhoff, 1972; Borden and Margoliash, 1975). The peptides of C_3 (residues 81–104) were arranged by the data alone.

Sequence Studies on Webster-Swiss Mouse Cytochrome c. Webster-Swiss mouse cytochrome *c* was digested with trypsin and the resulting peptides were chromatographed on Dowex-50. For some of these peptides further purification was necessary and was accomplished utilizing the techniques of thin-layer peptide mapping, high voltage paper electrophoresis, and paper chromatography. The cyanogen bromide fragment of mouse cytochrome *c* (residues 66–80) was also isolated. Peptides representing the entire amino acid sequence of Webster-Swiss mouse cytochrome *c* were, in general, examined by the Edman degradation. The relative positions of these peptides in the polypeptide chain were decided by comparison with the amino acid sequences of other mammalian cytochromes *c* (Dayhoff, 1972; Borden and Margoliash, 1975).

The amino-terminal pentapeptide was subjected to partial acid hydrolysis and the products (purified by paper electrophoresis) were a tripeptide (Val-Glu-Lys), free aspartic acid, and a ninhydrin negative acidic material. This material was detected by the hypochlorite–iodide–starch reaction (Easley, 1965), contained glycine, and corresponded to *N*-acetylglycine.

The tryptic peptide covering residues 80–86 could not be analyzed by Edman degradation because of complete loss to the organic phase at the first cycle. It was treated with Braunitzer reagent III (Braunitzer et al., 1971) and was then carried successfully through five cycles of the degradation.

The complete sequence of mouse cytochrome *c* is shown in Figure 1. The points of cleavage which gave rise to the peptides of both Webster-Swiss and BALB/c mouse cytochrome *c* are also shown.

Sprague-Dawley Rat Cytochrome c. This cytochrome *c* was subjected to the same cleavage procedures as BALB/c mouse cytochrome *c*. The homologous peptides (with minor exceptions) were isolated, primarily by paper electrophoresis and chromatography. The amino acid composition and N termini were determined for all of the isolated rat peptides. These peptides represent the entire molecule. These data are identical with those for mouse cytochrome *c*.

The net charge at pH 6.45 was calculated for almost all of the rat cytochrome *c* peptides from high voltage electrophoresis data. Except for Glx at residue positions 31 and 36, all the Glx and Asx residues are represented in those peptides whose net charges were determined. The intact cytochromes *c* of rat and mouse also behaved identically upon electrophoresis in starch gel at pH 5.5. (This system distinguished readily between oxidized and reduced cytochrome *c*, which differ by only a single change.) These results are consistent with identity between the acid and amide residues of mouse and Sprague-Dawley rat cytochrome *c*.

Studies on Wistar Rat and Guinea Pig Cytochromes c. Tryptic and chymotryptic digestions of those two cytochromes as well as Webster-Swiss mouse cytochrome *c* were subjected to thin-layer peptide mapping procedures. The peptide maps of several different tryptic digests of all three proteins were also indistinguishable, as were the maps of several different chymotryptic digests. To test whether any electrophoretic or chromatographic differences in mobility could be detected, peptide mapping of mixtures of equal amounts of tryptic or chymotryptic digests of all possible pairs of the three proteins was performed. No new peptide spots were observed, indicating complete mapping identity.

Discussion

Sequence Identity among Rodent Cytochromes. The conclusion that the cytochromes *c* of the two strains of mice are identical was based on two independent determinations of the primary structure. However, the evidence presented for the identity of the amino acid sequences of the cytochromes *c* from the mouse, the two strains of rat, and guinea pig cannot be considered final. It is, nevertheless, extremely unlikely that any sequence differences could have escaped detection when peptide maps of tryptic and chymotryptic digests are all identical for the three proteins. This is especially true in the case of rat cytochrome *c*, since the amino acid compositions of the chymotryptic peptides representing the entire molecule were also determined. For such small proteins as cytochrome *c*, the amino acid composition of the whole protein can readily be established to a precision of better than one residue by appropriate analyses. Furthermore, peptide maps of mixtures of digests from two proteins are highly sensitive to small changes in mapping parameters and peptide maps are ideal for the

observation of a small number of differences between related proteins. Peptide maps have in fact easily found the threonine–serine interchange that occurs between horse and donkey cytochromes *c*,[2] as well as the threonine–serine, glycine–lysine, and glycine–threonine interchanges between the horse and hog proteins (Stewart and Margoliash, 1965). The type of sequence variation that might escape detection would be one in which two residues exchange positions within the same peptide in both the chymotryptic and the tryptic fragments representing that segment of the protein. The smaller the protein and the larger the number of fragments obtained the less likely such a contingency. Amino acid composition identity would also be maintained if such an exchange occurs in distant parts of the sequence, but this would lead to peptide map parameter changes in two separate peptides.

Many rodents are likely to have the same cytochrome *c*, barring required functional adaptations, such as those which possibly underlie the remarkable three residue difference between hog and hippopotamus cytochrome *c*.[3] This prediction arises from the fact that guinea pigs belong to a different suborder (Hystricomorpha) from rats and mice (Myomorpha) and are presumed to be of South American origin whereas rats and mice are of Old World origin (Romer, 1966). This would be similar to the situation with some artiodactyls (hog, sheep, and cow), some carnivores (dog and panda), and two camels and a whale (camel, guanaco, and California grey whale), three groups of species which, notwithstanding considerable evolutionary divergence within the groups, each have the same cytochrome *c*.

Immunological Considerations. The amino acid sequence of mouse cytochrome *c* is important for the study of molecular parameters of antigenicity employing different cytochromes *c* as model globular protein antigens. The fact that it differs from rabbit cytochrome *c* by only two residues is a particularly favorable situation because of the simplicity of the immunological response to the mouse protein by rabbits and vice versa (Reichlin, 1975).

Generation Time Hypothesis. Our findings permit a test of the generation time hypothesis of Laird et al. (1969) and Kohne (1970). According to their hypothesis, molecular evolution is faster in organisms with short generations (e.g., rodents) than in organisms with long generations (e.g., primates). It is clear, however, from our work that the rodent cytochrome *c* sequences are highly conserved. The same cytochrome *c* sequences occur in quite different rodents; furthermore, the rodent sequence differs by a minimum of 2 base changes from rabbit cytochrome *c* and 10 base changes from human cytochrome *c*. If one assumes that, since the point at which those lines diverged, the average generation times have been much shorter on the lines leading to rodents and rabbits than on the line leading to humans, then the obvious conclusion is that these data do not support the generation-time hypothesis. In fact, cytochrome *c* appears to have experienced faster evolution in primates than in rodents.

A more rigorous analysis of relatives rates of cytochrome *c* evolution in long and short generation-time organisms requires a calculation of several factors. These factors include the relative times of divergence, the phyletic[4] distances of these cytochromes *c* from their common mammalian ancestral gene, and the generation-time differential between these lineages. For this analysis one may consult Carlson (1975) and Wilson et al. (1977). For the phylogenetic tree and table of minimal mutation distance on which that analysis is based, see the paragraph at the end of this paper concerning supplementary material.

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Supplementary Material Available

Additional information regarding the experimental procedures involved with cytochrome *c* and peptide purification, amino acid compositions, and sequence data summaries for BALB/c and Webster-Swiss mouse cytochrome *c* peptides, amino acid composition, and net charge at pH 6.5 for Sprague-Dawley rat peptides, and solid phase sequence data. Also included is a phylogenetic tree for mammalian cytochromes *c*, constructed by the Farris (1972) method, as well as a table of minimum mutational distances from which this tree was constructed (27 pages). Ordering information is given on any current masthead page.

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[2] Walasek, O., and Margoliash, E., unpublished.

[3] Thompson, R. B., Tarr, G. E., and Margoliash, E., unpublished.

[4] Phyletic distance is the estimated number of substitutions that have accumulated in the evolutionary descent of a macromolecular sequence. This estimate is obtained by phylogenetic methods, which reconstruct an evolutionary tree from cytochrome *c* sequences of present-day organisms (Wilson et al., 1977).

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