References and Notes

1. F. H. Rigler, in *The Phosphorus Handbook*, A. M. Beeton, Ed. (Wiley, New York, 1972).
2. D. R. S. Lean and F. H. Rigler, in preparation.
3. D. S. Riggs, *The Mathematical Approach to Physiological Problems* (Williams & Wilkins, Baltimore, 1963), pp. 201–215.
4. E. T. Gjessing, *Nature* **208**, 1081 (1965).
5. E. H. McConkey, *Methods Enzymol.* **12** (part A), 630 (1967).
6. N. Ogura, *Limnol. Oceanogr.* **15**, 476 (1970); W. H. Sutcliffe, Jr., E. R. Baylor, D. W. Menzel, *Deep-Sea Res.* **10**, 233 (1963); P. J. Wangersky, *Amer. Sci.* **53**, 358 (1965).
7. Zooplankton do not contribute to this form. Similar kinetic data were obtained for lake water and lake water that had been passed through a 56-μm filter to remove major zooplankton.
8. F. H. Rigler, *Limnol. Oceanogr.* **9**, 511 (1964).
9. J. D. H. Strickland and T. R. Parsons, *Bull. Fish. Res. Board Can.* **167**, 1 (1968).
10. D. R. S. Lean and D. W. Schindler, in preparation.
11. G. E. Fogg, *Ergeb. Limnol.* **5**, 11 (1971).
12. N. J. Antia, C. D. McAllister, T. R. Parsons, K. Stephens, J. D. H. Strickland, *Limnol. Oceanogr.* **8**, 166 (1963); R. E. Johannes, *ibid.* **9**, 224 (1964); E. J. Juenzler, *J. Phycol.* **8**, 7 (1970).
13. W. D. Watt and F. R. Hayes, *Limnol. Oceanogr.* **8**, 276 (1963).

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* Present address: Biogeochemical Limnology Subdivision, Lakes Research Division, Canada Centre for Inland Waters, Burlington, Ontario.

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Reaction of Hydrated Electrons with Ferricytochrome c

Abstract. *The reaction of ferricytochrome c with hydrated electrons produced at pH 6.8 by radiolysis with electron pulses lasting 50 to 1000 nanoseconds has a specific rate constant of 5.5×10^{10} liter mole^{-1} sec^{-1} for the formation of the primary adduct. By using appropriate wavelengths, another fast, consecutive process was demonstrated, with the pure first order rate constant 1.0×10^5 sec^{-1}. Its characteristics agree with it being an intramolecular process within the enzyme. Approximately 50 percent of all electrons which add to ferricytochrome c end in forming ferrocytochrome c.*

The development of very fast pulse radiolysis techniques in aqueous solutions makes possible the quantitative production of simple radicals, such as hydrated electrons, e^-_{aq}; hydrogen atoms, H; and hydroxyl radicals, OH. These have only thermal energy and react in a specific and selective way with solutes. The reactions of cytochrome c (*1*) with e^-_{aq} or H (*2*) allow one to demonstrate the effects of one-electron equivalent reagents on oxidation reduction enzymes down to the diffusion-controlled time limit. The role of the protein in the electron equivalent transfer can also be demonstrated.

Using such methods, we studied the reactions of these radicals with the hydrolytic enzyme, ribonuclease, and showed that primary, very fast, nonselective addition to the protein was followed by consecutive first order intramolecular processes of radical transfer, affecting specifically and selectively the divalent sulfur and aromatic amino acid components in a linked process. For the hydrolytic enzyme, not designed for redox processes, there were irreversible chemical changes and enzyme inactivation (*3*).

Using spectrophotometric techniques, we studied the reaction of H atoms (produced by nanosecond pulse radiolysis) with Fe(III)-cytochrome c in aqueous solution at *p*H 2.5 and 6.5 (*4*). We found that H atoms add to the enzyme with the second order rate constant $k \cong 2 \times 10^{10}$ liter mole $^{-1}$ sec^{-1}. The primary addition was followed by three consecutive intramolecular processes, with first order rate constants of approximately 1×10^5, 2×10^4, and 2×10^3 sec^{-1}. The spectroscopic characteristics of the first two processes were consistent with the transfer of an electron equivalent through the protein to the Fe(III) moiety, to yield Fe(II). The slowest, third process could be attributed to configurational changes

We also reported briefly our results on the reaction of e^-_{aq} with Fe(III)-cytochrome c, for which we obtained $k \cong 4 \times 10^{10}$ liter mole^{-1} sec^{-1}. Hydrogen atoms do not absorb energy in the spectroscopic range of cytochrome c (370 to 650 nm). Above about 500 nm, e^-_{aq} absorbs sufficiently to complicate observations on the transition Fe(III) → Fe(II). Accordingly, processes involving e^-_{aq} are somewhat harder to elucidate than those involving H.

Land and Swallow (*5*) studied the reaction of e^-_{aq} with Fe(III)-cytochrome c by using pulses of approximately 200 to 400 rads and cytochrome c concentrations of 4×10^{-6} to $3 \times 10^{-5}M$. At a *p*H of about 7 they found $k \cong 2 \times 10^{10}$ liter mole^{-1} sec^{-1}. In their system, $0.1M$ formate buffer was present, and they corrected for its reaction with the radicals produced by irradiation. Their results at *p*H 7 were interpreted as showing that within 150 nsec Fe(III) was converted to Fe(II), the rate of reduction being equal to the rate of addition.

Pecht and Faraggi (*6*) reported similar experiments, with 100-nsec pulses (approximately 400 to 500 rads) at cytochrome c concentrations of 5×10^{-7} to $3 \times 10^{-6}M$, but they found $k \cong 1.5 \times 10^{11}$ liter mole^{-1} sec^{-1}. The interpretation of their results is complicated by the fact that at the low concentrations and relatively high dose employed, there are more e^-_{aq} available than Fe(III)-cytochrome c. They assumed that at the very high rate constants they calculated, e^-_{aq} reacts directly with the Fe(III) moiety.

For the experiments reported here we used deaerated aqueous solutions of Fe(III)-cytochrome c (Sigma type IV, 95

Table 1. Apparent first order rate constants (k_{obs}, in sec^{-1}) and calculated second order rate constants (k_1, in liter mole^{-1} sec^{-1}) for the reaction between ferricytochrome c and e^-_{aq} at *p*H ~6.8.

Cytochrome c $\times 10^{-5}M$	650 nm		580 nm		535 nm		520 nm		510 nm		370 nm	
	$k_{obs} \times 10^5$	$k_1 \times 10^{10}$	$k_{obs} \times 10^5$	$k_1 \times 10^{10}$	$k_{obs} \times 10^5$	$k_1 \times 10^{10}$	$k_{obs} \times 10^5$	$k_1 \times 10^{10}$	$k_{obs} \times 10^5$	$k_1 \times 10^{10}$	$k_{obs} \times 10^5$	$k_1 \times 10^{10}$
5	27 ± 3	5.4 ± 0.6	27 ± 3	5.4 ± 0.6	30	6.0	30	6.0				
1	6.2 ±1.7	6.2 ± 1.7	5.5 ± 1.5	5.5 ± 1.5	6.8 ± 1.7	6.8 ± 1.7	5.1 ± 1.4	5.1 ± 1.4	5.3 ± 0.5	5.3 ± 0.5	4.2 ± 0.2	4.2 ± 0.2
0.5	2.8 ± 1.7	5.6 ± 0.8	3.0 ± 0.7	6.0 ± 1.4							2.5 ± 0.3	5.0 ± 0.6
0.25	2.4 ± 0.4	[10]	2.5 ± 0.8	[10]	2.2 ± 0.3	[9]	2.3 ± 0.3	[9]			1.2 ± 0.2	[4.8]

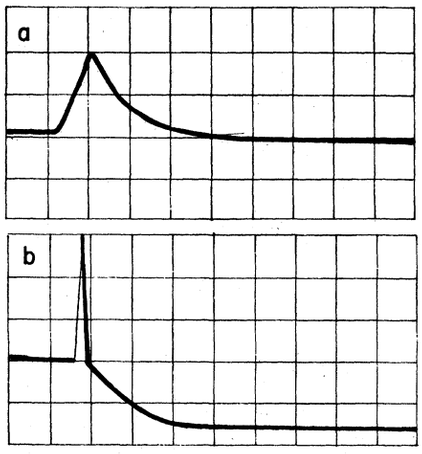

Fig. 1. Traces of oscilloscope pictures obtained at 580 nm, with 2.5-Mev electrons, $5 \times 10^{-5}M$ Fe(III)-cytochrome c, *p*H ~ 6.8. (a) Pulse duration, 300 nsec; dose per pulse, 1000 rads; sweep rate, 500 nsec per division; V_0, 100 mv; 5.0 mv per division. (b) Pulse duration, 500 nsec; dose per pulse, 1500 rads; sweep rate, 10 μsec per division; V_0, 100 mv; 2.0 mv per division. The initial rise shows the formation of e^-_{aq} during the pulse. The following fast decay is due to the disappearance of the electron and the formation of the primary adduct. The final slow reaction, a first order intramolecular process, is particularly clear in (b), bringing the absorbancy below the base line.

to 100 percent, prepared without the use of chloroacetic acid). Tertiary butanol (always 10^4 times the cytochrome c concentration) was present to scavenge OH radicals. The *t*-butanol radical has a low reactivity (*7*) and was found not to affect the results. The *p*H was set in the range 6.4 to 6.9 with NaOH or $HClO_4$. Special attention was paid to the optical conditions. Spectra were taken with a bandwidth of 2 nm, an optical path of 2 cm, and appropriate filters to remove stray light. Details of the experimental arrangements have been described (*3*). Pulses of electrons (2.5 to 5 Mev) lasting 50 to 1000 nsec were applied. The dose per pulse was 300 to 3000 rads. All the kinetic data reported were obtained from the first pulse given to a fresh solution. From an inspection of the oscilloscope traces, the primary reaction of e^-_{aq} with Fe(III)-cytochrome c was found to be a second order process with $k = (5.5 \pm 1.5) \times 10^{10}$ liter mole^{-1} sec^{-1}, independent of wavelength. Table 1 shows the pseudo first order constants, and the second order constants obtained from them. At cytochrome c concentrations of 1×10^{-5} and $5 \times 10^{-5}M$ the rate constants do not change in the dose range 350 to 1000 rads, and at $5 \times 10^{-6}M$ cytochrome c they do not change from 350 to 600 rads. The yield of electrons is approximately $3 \times 10^{-9}M$ per rad. With a lower concentration of cytochrome c, $2.5 \times 10^{-6}M$, and doses of 350 to 1000 rads, a pseudo first order process is observed. Under these conditions the concentration of e^-_{aq} is greater than or equal to that of cytochrome c. Nevertheless, if one divides by the concentration of cytochrome c, an apparent second order constant of approximately 1×10^{11} liter mole^{-1} sec^{-1} is obtained, leading to an apparent increase in the rate of constant over the constant value at higher concentration. Even at this lowest concentration at 370 nm (where e^-_{aq} does not appreciably absorb), the lower rate constant is obtained.

In Table 2 the results obtained for the consecutive process are shown. This reaction—a strictly first order process—is observed most clearly at 580 nm; it is also detectable at 560 and 535 nm. Its rate constant, 1×10^5 sec^{-1}, is unaffected by changes in the total dose or the cytochrome c concentration, unlike that of the primary addition process. Because it is a fast process, it cannot be separated at lower cytochrome c concentrations, where the slow rate of the primary process causes the reactions to overlap. At $1 \times 10^{-5}M$ cytochrome c, the two processes can be separated with difficulty, but at $5 \times 10^{-5}M$ they can be separated readily.

Table 2. First order rate constant of the intramolecular reaction, measured at 580 nm, *p*H ~ 6.8.

Cytochrome c $\times 10^{-5}M$	k ($\times 10^5$ sec^{-1})			
	600 rads	1000 rads	1500 rads	3000 rads
5	1.0	1.0	1.0	1.1
1		1.1		

The measurements at 580 nm (Fig. 1) gave clear-cut results. Over the spectral region 500 to 650 nm absorption due to Fe(III)-cytochrome c, Fe(II)-cytochrome c, e^-_{aq}, and the intermediate or intermediates initially formed by the addition of e^-_{aq} to cytochrome c is observed. A complex temporal sequence occurs with consecutive decreases and increases in absorption. At the Fe(II)-cytochrome c absorption peak at 550 nm, for instance, as long as the initial concentration of Fe(III)-cytochrome c is high enough, the changes due to the disappearance of e^-_{aq} and the formation of intermediates followed by Fe(II) appear to compensate exactly. At 580 nm the extinction coefficient of Fe(III) is greater than that of Fe(II) (*1*, *8*), and the results give clearly separable events.

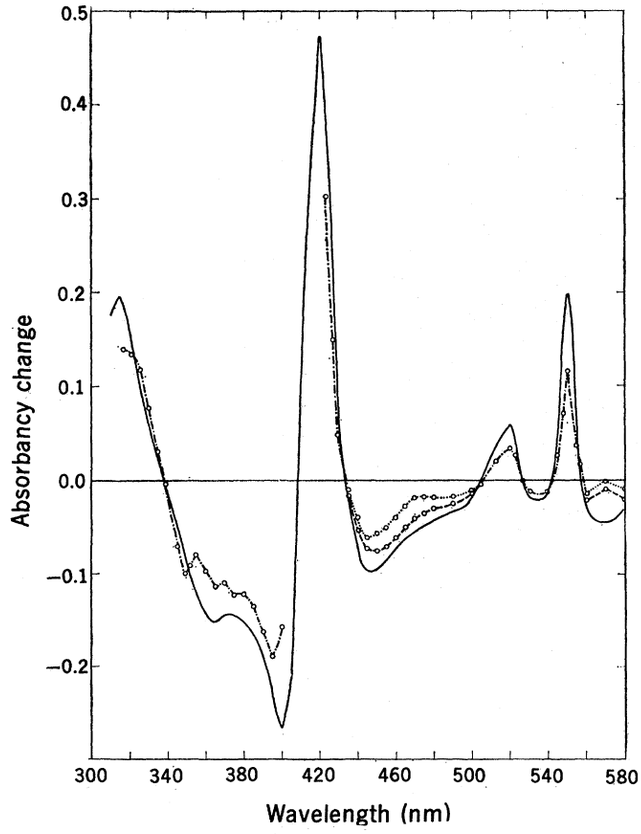

Fig. 2. Difference absorption spectrum of $2 \times 10^{-5}M$ Fe(III)-cytochrome c solution at *p*H 6.8. The continuous line was calculated by assuming full reduction to Fe(II)-cytochrome c by e^-_{aq} and H produced by the pulse. (Dotted line) Measurements 5 μsec after a pulse of approximately 1500 rads. (Dashed line) Measurements 30 μsec after a pulse of approximately 1500 rads. (Dotted and dashed line) Results at 5 and 30 μsec coincide.

Thus we observe, after the initial fast rise due to the formation of e^-_{aq}, two consecutive processes: first, a fast addition of e^-_{aq} to Fe(III)-cytochrome c, and second, the resultant slower formation of Fe(II)-cytochrome c. There may be faster intramolecular processes. With our present procedures, only the process with $k = 10^5$ sec^{-1} can be determined. The observations between 320 and 580 nm are summed up in Fig. 2.

Under the present experimental conditions the yield of e^-_{aq} is approximately six times that of H. Reactions due to H are thus of secondary importance.

From the results at 550 and 580 nm we can estimate approximately the fraction of the electrons which, having all added to the enzyme, reach the iron moiety and reduce Fe(III) to Fe(II). From the known extinction coefficients of Fe(III), Fe(II), and e^-_{aq}, the yield of e^-_{aq}, and the resultant absorbancy change, we calculate that at 550 nm 45 ± 5 percent and at 580 nm 60 ± 12 percent of the electrons produce reduction of the central Fe atom.

NORMAN N. LICHTIN*
AVIGDOR SHAFFERMAN, GABRIEL STEIN
Department of Physical Chemistry, Hebrew University, Jerusalem, Israel

References and Notes

1. E. Margoliash and A. Schejter, *Advan. Protein Chem.* **21**, 114 (1966).
2. G. Czapski, N. Frohwirth, G. Stein, *Nature* **207**, 1191 (1965).
3. N. N. Lichtin, J. Ogdan, G. Stein, *Radiat. Res.* **47**, 349 (1971); *Biochim. Biophys. Acta* **263**, 14 (1972); *ibid.* **276**, 124 (1972).
4. ———, *Israel J. Chem.* **9**, 579 (1971).
5. E. J. Land and A. J. Swallow, *Arch. Biochem. Biophys.* **145**, 365 (1971).
6. I. Pecht and M. Faraggi, *Fed. Eur. Biol. Soc. Lett.* **13**, 221 (1971).
7. G. Scholes and M. Simic, *J. Phys. Chem.* **68**, 1738 (1964); M. Simic, P. Neta, E. Hayon, *ibid.* **73**, 3794 (1969).
8. E. Margoliash and N. Frohwirth, *Biochem. J.* **71**, 559 (1959).
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* On leave from the Department of Chemistry, Boston University, Boston, Mass.

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DNA Complementary to Ribosomal RNA: Relation between Genomic Proportion and Ploidy

Abstract. *Ten* Nicotiana *species were assayed for the proportion of DNA that is complementary to ribosomal RNA. This proportion varies from 0.27 to 0.9 percent, with tetraploid species having lower values than the diploid species. The tetraploid species have about twice as much DNA per cell as do diploid species. Thus, the absolute number of genes for ribosomal RNA varies less than the proportion of complementary DNA. Further, the number of genes for the RNA in 80S ribosomes varies less among species than does that for the RNA in 70S ribosomes. The data indicate that loss of DNA complementary to ribosomal RNA is associated with tetraploidy in the genus* Nicotiana.

Organisms differ in the proportion of rDNA, the DNA that is complementary to ribosomal RNA (rRNA). The published values vary from 0.011 percent of total DNA for *Neoceratodus forsteri* to almost 4 percent for certain yeasts (*1, 2*), and in higher plants the proportion ranges from 0.02 percent for *Helianthus ruberosis* to 3 percent for *Cucurbita maxima* (*3–5*). A study was undertaken to determine whether this proportion might be similar for species within a genus and thus perhaps of phylogenetic or phyletic significance. Data for ten species in the genus *Nicotiana* are shown in Table 1 along with their position in the phylogenetic scheme devised for this genus by Goodspeed (*6*). Although the genomic proportion of rDNA varies considerably among the species (from 0.27 to 0.90 percent), it does not agree with the taxonomy of the genus as deduced from other characteristics. There is a substantial difference between species in all three subgenera examined and even between the species *N. paniculata* and *N. glauca* belonging to the same section. Thus, genomic proportion of rDNA appears to be a poor indicator of species relatedness, in agreement with the conclusion drawn from a similar study of the genus *Cucurbita* (*5*).

A relation between proportion of rDNA and chromosome number of the species becomes apparent, however, when the data in Table 1 are examined further. The genus *Nicotiana* contains species with either 24 or 48 chromosomes (or derivatives of these numbers), which we shall refer to, respectively, as diploid and tetraploid species. The tetraploid species examined have a genomic proportion of rDNA ranging from 0.27 to 0.43 percent, whereas the diploid species all have a higher value (0.67 to 0.90 percent). Thus, the DNA's of tetraploid species appear to have a lower proportion of rDNA than do the diploid species in this genus. *Nicotiana glauca* is a seeming exception to this rule because it has a relatively low proportion of rDNA and is reported to be a diploid species (*6*). In matter of fact this proved not to be an exception, because the plants used for DNA extraction were a local wild isolate that proved to be tetraploid when root-tip mitotic figures and flower-bud meiotic figures were examined.

The DNA's of several plant species such as pumpkin and Chinese cabbage, which have a relatively high genomic proportion of rDNA, also display a

Table 1. Genomic proportion of rDNA and chromosome number for ten *Nicotiana* species. Chromosome numbers (CN) and species arrangement are from Goodspeed (*6*). The values for rDNA were obtained by incubating an excess of ^{3}H-labeled tobacco leaf rRNA with 10 to 60 μg of alkali-denatured nuclear DNA embedded in nitrocellulose membranes; incubations were done in medium containing 0.3*M* sodium chloride and 0.03*M* sodium citrate at 68°C for 18 hours (*14*). Each value represents the average of at least two (usually more) determinations with independently prepared reagents. The listed values are within 10 percent of the most divergent determinations. Reagents were prepared as described (*7, 15*). Nuclear DNA was prepared from a 1000*g* pellet of a leaf macerate. The pellet was washed with Triton X-100 (Rohm and Haas), treated with ribonuclease and Pronase, and extracted with phenol; the resultant material was purified by preparative isopycnic banding in cesium chloride. Labeled rRNA was prepared from a macerate of leaf tissue that had been exposed to [^{3}H]uridine (*17*); the material extracted with phenol was purified by successive precipitations with ethanol and 2*M* lithium chloride (*18*). The different rRNA preparations were monitored for purity by polyacrylamide gel electrophoresis before use and had 5000 to 6000 count/min per microgram. Most populations of *N. glauca* have 24 chromosomes, but the plants used in this study had 48.

Genus *Nicotiana*	rDNA (% of total DNA)	CN
Subgenus Tabacum		
Section Genuinae		
Species *tabacum*	0.28	48
Section Tomentosae		
Species *glutinosa*	0.67	24
Subgenus Rustica		
Section Rusticae		
Species *rustica*	0.27	48
Section Paniculatae		
Species *paniculata*	0.80	24
Species *glauca*	0.32	24 (48)
Subgenus Petunioides		
Section Alatae		
Species *sylvestris*	0.90	24
Section Acuminatae		
Species *acuminata*	0.67	24
Section Bigelovianae		
Species *bigelovii*	0.32	48
Section Suaveolentes		
Species *benthamiana*	0.33	38
Species *occidentalis*	0.43	42