The Reduction of Cytochrome *c* by Free Radicals in Irradiated Solutions

By L. K. MEE

Department of Radiotherapeutics, University of Cambridge

AND G. STEIN

Department of Physical Chemistry, Hebrew University, Jerusalem

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The mechanism of reduction processes, both enzymic and non-enzymic, has recently been the subject of numerous investigations, and evidence about the single steps involved has become available. A method of reduction based on the action of ionizing radiations on suitably constructed systems (Day & Stein, 1951*a, b*) shows some specific features of interest.

In aqueous solutions, not containing solutes possessing heavy atoms, ionizing radiations are absorbed in approximate proportion to the masses of the constituents. Thus in dilute solutions direct action of the radiation on the solutes may be disregarded and absorption will be mainly by the solvent water. If one considers the main effect to be the splitting of the solvent molecules in the following way (Weiss, 1944):

one may interpret the chemical changes observed as the effects of the H atoms and OH radicals thus obtained. Considering the latter, in a solution containing a relatively small concentration of one substance, S, and a relatively much higher concentration of another, AH₂, which may be, for example, ethanol, benzoate or succinate, the OH radicals will react preferentially with AH₂, yielding the organic free radicals $\dot{\text{A}}\text{H}$:

The radicals $\dot{\text{A}}\text{H}$ may act as either oxidizing or reducing agents on the substance S (Swallow, 1953).

Thus it was shown that when methylene blue or other dyes are irradiated in dilute aqueous solution with X- or γ -rays in the absence of oxygen but in the presence of relatively high concentrations of benzoate or succinate the dye can be completely reduced in an entirely reversible manner. If ethanol is used similar results are obtained, yet some irreversible decoloration occurs as well (Day & Stein, 1951*a, b*; Stein, 1952, 1955; Day, 1952).

If coenzyme I is used, then in the absence of oxygen but in the presence of benzoate or succinate no reduction occurs in irradiated solutions. If,

however, ethanol is used reduction occurs. The product, though closely related to normal reduced diphosphopyridine nucleotide (DPNH), remains enzymically inactive (Swallow, 1953, 1955; Stein & Swallow, 1954). Similar results were obtained with various DPN⁺ models (Stein & Swallow, 1954; Stein, 1955; Stein & Stiassny, 1955).

In view of these results it was of interest to carry out similar experiments on cytochrome *c*. These have been briefly referred to before (Stein & Swallow, 1954).

EXPERIMENTAL

The horse-heart cytochrome *c* used was prepared by Margoliash (1954). It was his fraction I, obtained at pH 7, containing 0.465% of iron. The millimolar extinction coefficient at 550 m μ . of the completely reduced form was 27.7, of the completely oxidized 9.2, measured in 0.1M phosphate buffer at pH 7.

All cytochrome *c* solutions were approx. 10⁻⁵M in permanganate-distilled water, and were evacuated with boiling at room temperature on an oil pump. The N₂ and H₂ used were purified O₂-free quality, supplied by British Oxygen Co. Ltd. In all cases the cytochrome *c* solutions were aerated before the absorption spectra were read.

Irradiations were carried out with 200 kv peak X-rays filtered through 1 mm. Cu and 1 mm. Al, and at pH 7. Optical densities were determined on a Beckman DU spectrophotometer and all values reported refer to 1 cm. cells. The heart-muscle enzyme system preparation used was obtained according to Keilin & Hartree (1947).

RESULTS

In Fig. 1*A* the absorption curve of the original 10⁻⁵M cytochrome *c* solution before irradiation is shown. Changes in the spectrum on irradiation were in four regions: (a) a shift in the maximum from 410 to 415 m μ .; (b) increased absorption at 520 m μ .; (c) increased absorption at 550 m μ .; (d) increased absorption at 600 m μ . Changes (a), (b) and (c), but not (d), appear on reduction of cytochrome *c* enzymically or with dithionite. When a 10⁻⁵M solution of cytochrome *c* is irradiated in the absence of O₂ but in the presence of either

Fig. 1. Absorption spectrum of approx. 10^{-5} M cytochrome *c* solution (1 cm. cell). *A*, Before irradiation; *B*, after irradiation with 60 000 r. in the presence of 0.1 M ethanol, in the absence of O_2 , followed by reoxidation.

Fig. 2. Absorption spectrum of approx. 10^{-5} M cytochrome *c* solution (1 cm. cell) after irradiation with 30 000 r. of X-rays in the presence of 0.1 M benzoate (●), 0.1 M succinate (○), 0.1 M ethanol (×), in the absence of O_2 .

sodium benzoate or sodium succinate (0.1M) with an X-ray dose of 30 000 r., the spectra shown in Fig. 2 are obtained. These show changes (a), (b) and (c) but not (d), thus corresponding to reduced cytochrome *c*. To ascertain whether the product behaves as reduced cytochrome *c*, it was allowed to react with the heart-muscle preparation. With this it could be repeatedly reoxidized by the cytochrome-oxidase and reduced by the succinic-dehydrogenase system, the reoxidized form yielding the unchanged spectrum of the starting material. Thus this method appears to produce the normal reduced cytochrome *c*.

When ethanol (0.1M) was used instead of benzoate or succinate and a dose of 30 000 r. was given, the spectrum shown in Fig. 2 was obtained. There is less of a shift from 410 to 415 m μ ., and less increase at 520 and 550 m μ .. However, at 600 m μ ., a new absorption band appears in the ethanolic irradiated solution. The irradiated solution can be reoxidized with, for example, ferricyanide and the spectrum shown in Fig. 1B is then obtained, in which the band at 600 m μ .. persists. This product is thus not the one formed on enzymic reduction.

With the amount of cytochrome *c* in the solutions employed 15 000 r. was sufficient for complete reduction. It was of interest to see the effect of continued irradiation. The results of irradiation with 60 000 r., compared with those of Fig. 2, show that in the presence of benzoate changes (a), (b) and (c) appropriate to the genuine reduced form

Fig. 3. Dependence of optical density of cytochrome *c* solution (approx. 10^{-5} M, in 1 cm. cell, measured at 600 m μ .) on radiation dose. Irradiations carried out in the presence of 0.1M ethanol, in the absence of O₂. Measurements in reaerated solutions.

Fig. 4. Absorption spectrum of approx. 10^{-5} M cytochrome *c* solution (1 cm. cell) after irradiation with 45 000 r. of X-rays in the presence of approx. 10^{-5} M-H₂ (●), 0.1M benzoate (○), 0.1M ethanol (x), in the absence of O₂.

persist, so that in the presence of benzoate reduced cytochrome *c* is not affected on further irradiation. In particular no band appears at 600 m μ . In the presence of ethanol, however, continued irradiation results in the disappearance of peaks at 520 and 550 m μ ., but the band at 600 m μ . increases in intensity.

The dependence of optical density on radiation dose in this band is shown in Fig. 3 to be approximately linear. Methanol, ethanol and *n*-butanol (0.1 M) all produce similar effects.

When, instead of ethanol, succinate or benzoate, the solution is saturated with H₂ (thus giving approx. 10⁻³ M concentration of the gas in solution) the results shown in Fig. 4 are obtained at a dose of 45 000 r. The results in H₂-containing solutions thus resemble those obtained in ethanolic ones.

When irradiations were carried out in solutions containing only N₂ a dose of 45 000 r. produced no shift to 415 m μ ., no peaks at 520 or 550 m μ ., but the band at 600 m μ . did appear. By contrast, in O₂-containing solutions at this dose no apparent change occurs. At the same dose in the presence of ethanol not only the band at 600 m μ . but also changes (a), (b) and (c) typical of the normal reduction product appear.

DISCUSSION

The reactions observed in the present system are in fact based on the action of organic free radicals derived from benzoate, succinate or alcohols according to reaction 2. They show that these organic radicals act as reducing agents towards cytochrome *c*. As shown, reduced cytochrome *c* remains in its reduced state with continued action of these radicals, derived from benzoate or succinate. The reducing action of organic radicals derived from benzoate or succinate produces an enzymically active reduced form, which has a spectrum corresponding in all respects with the biological reduction product. There appears to be a specific difference between these radicals and those derived from methanol, ethanol or *n*-butanol. With coenzyme I and coenzyme I models the radicals derived from benzoate or succinate were found to be incapable of effecting reduction. The radicals derived from the alcohols did effect reduction, but in such solutions a product not normally obtained in biological systems was also

produced. The results obtained indicate that this unusual product can be formed, not only by the action of organic radicals such as CH₃.CH.OH but also on the action of H atoms in cytochrome *c*-containing solutions. In H₂-saturated solutions the reaction

occurs (Stein & Weiss, 1949).

The spectrum of this product on examination under the ocular spectroscope is similar to that of porphyrin *c* obtained by Hill & Keilin (1931) from cytochrome *c* in the presence of a reducer (SO₂), in the absence of O₂, in hydrochloric acid solutions.

SUMMARY

1. Aqueous solutions of cytochrome *c* (10⁻⁵ M) on irradiation with X-rays in the absence of O₂ and in the presence of 0.1 M benzoate or succinate yielded reduced cytochrome *c* identical with the product of enzymic reduction.

2. In the presence of methanol, ethanol or *n*-butanol a product not identical with that resulting on enzymic reduction is also obtained.

3. This same unusual product is formed in the presence of H₂. Its spectrum is similar to that of porphyrin *c*.

4. The results are interpreted in terms of the reactions of organic free radicals produced in these systems.

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REFERENCES

- Day, M. J. (1952). *Disc. Faraday Soc.* **12**, 280.
- Day, M. J. & Stein, G. (1951a). *Nature, Lond.*, **168**, 644.
- Day, M. J. & Stein, G. (1951b). *Nucleonics*, **8**, (2), 34.
- Hill, R. & Keilin, D. (1931). *Proc. Roy. Soc. B*, **107**, 286.
- Keilin, D. & Hartree, E. F. (1947). *Biochem. J.* **41**, 503.
- Margoliash, E. (1954). *Biochem. J.* **54**, 529.
- Stein, G. (1952). *Disc. Faraday Soc.* **12**, 227.
- Stein, G. (1955). *J. Chim. phys.* **52**, 634.
- Stein, G. & Stiassny, G. (1955). *Nature, Lond.*, **176**, 734.
- Stein, G. & Swallow, A. J. (1954). *Nature, Lond.*, **173**, 937.
- Stein, G. & Weiss, J. (1949). *J. chem. Soc.* p. 3245.
- Swallow, A. J. (1953). *Biochem. J.* **54**, 253.
- Swallow, A. J. (1955). *Biochem. J.* **61**, 197.
- Weiss, J. (1944). *Nature, Lond.*, **153**, 748.