Selenium as a Catalyst for the Reduction of Cytochrome *c* by Glutathione†

Orville A. Levander,* Virginia C. Morris, and Darla J. Higgs

ABSTRACT: Selenite at 10^{-5}–10^{-6} M is an effective catalyst for the reduction of cytochrome *c* by glutathione (GSH). Selenocystine was more effective and selenate less effective than selenite. Selenomethionine had no catalytic effect. A strong catalysis of selenite on the reduction of cytochrome *c* was still observed when mercaptoethylamine or cysteine replaced GSH as the thiol, but smaller catalytic effects of Se were seen when thioglycolate or mercaptoethanol was used in lieu of GSH. Cysteine was a more active reductant for cytochrome *c* than GSH either in the presence or absence of Se. Dithiothreitol and 2,3-dimercaptopropanol were both potent reducing agents for cytochrome *c* without Se, but a catalytic effect due to Se could still be demonstrated with these dithiols. Cyanide at 5×10^{-6} M caused a 50% inhibition in Se-catalyzed reduction of cytochrome *c* by GSH. Selenocyanate was a relatively poor catalyst for the reduction of cytochrome *c* by GSH. Cd^{2+} and Hg^{2+} were moderately good inhibitors of Se-catalyzed reduction of cytochrome *c* by GSH, but arsenite had little or no inhibitory effect even at levels as high as 10^{-2} M. A mechanism for the selenite-catalyzed reduction of cytochrome *c* by GSH is presented which involves the formation of a selenopersulfide intermediate. Selenium may act *in vivo* as selenopersulfide to facilitate the transfer of electrons from sulfhydryl groups to cytochrome *c*.

The use of selenium as a catalyst in Kjeldahl digestions is perhaps one of the most widespread applications of this element in biochemistry (Patel and Sreenivasan, 1948). Similarly, Se as a catalyst finds many uses in various industrial processes such as oxidations, hydrogenations, and isomerizations (Kollonitsch and Kline, 1963). A spot test for Se based on the catalytic effect of elemental Se on the reduction of Methylene Blue by sodium sulfide was reported by Feigl and West (1947). This procedure has been refined into a sensitive analytical method for the determination of trace amounts of Se (West and Ramakrishna, 1968). Gitler (1958) studied the Se-catalyzed reduction of Methylene Blue by cysteine and suggested that this might be an appropriate model for the biological function of Se. The ability of selenite to act as a catalyst for the reduction of Methylene Blue by a number of sulfhydryl amino acids has been investigated by Stanton (1962). In a review article, Schwarz, the discoverer of the nutritional significance of Se (Schwarz and Foltz, 1957), pointed out the similarity between the Se spot test described by Feigl and West and the diaphorase reaction (Schwarz, 1962). He speculated that Se might exert a catalytic effect in biological systems at the active site of a specific enzyme, or a group of specific enzymes. Recently, Levander *et al.* (1973) have shown that Se can accelerate the swelling of rat liver mitochondria in the presence of glutathione and certain other thiols. The catalytic effect of selenite on glutathione-induced swelling was found to be partially blocked by amytal or antimycin A and totally blocked by cyanide. This suggested that the glutathione–selenite swelling might be at least partly mediated at the level of cytochrome *c*. The results reported here demonstrate that Se is a highly effective catalyst for the reduction of cytochrome *c* by glutathione.

Methods and Materials

The system used to study the selenium-catalyzed reduction of cytochrome *c* by glutathione (GSH)[1] was similar to that employed by others to study the catalytic effect of GSSG on the reduction of cytochrome *c* by GSH (Froede and Hunter, 1970; Massey *et al.*, 1971). The basal medium consisted of 3×10^{-5} M cytochrome *c* plus 3.3×10^{-4} M GSH in 0.175 M KCl–0.025 M Tris-Cl buffer (pH 7.45). The reduction of cytochrome *c* was determined at room temperature by following the increase in absorbance at 550 nm in a Gilford spectrophotometer. That this change in absorbance at 550 nm actually represented reduction of cytochrome *c* was verified by comparing the visible spectrum of the reaction mixture with the published spectrum of reduced cytochrome *c*. The cytochrome *c* used was horse heart type III from the Sigma Chemical Co., St. Louis, Mo.[2] The selenoamino acids and thiols were also from Sigma except for cysteine which was from the Fisher Scientific Co., Fair Lawn, N. J. All other chemicals used were laboratory reagent grade.

Results

Selenium-Catalyzed Reduction of Cytochrome c by Glutathione. Figure 1 shows that addition of 10^{-6}–10^{-5} M selenite to the basal medium caused a progressive increase in the rate of reduction of cytochrome *c* by GSH. Reduction of cytochrome *c* by GSH alone was very slow. Oxidized glutathione at 3.3×10^{-3} M also effectively catalyzed the reduction of cytochrome *c* by GSH. Selenate at 10^{-4} M catalyzed the reduction of cytochrome *c* by GSH but at a slower rate (Table I). Selenomethionine at 10^{-4} M had essentially no effect on the reduction of cytochrome *c* by GSH. Selenocystine at 10^{-5} M was more active than the same concentration of selenite in accelerating

† From the Nutrition Institute, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland 20705. *Received June 18, 1973.* Presented at the 57th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1973.

[1] Abbreviations used are: GSH, glutathione; GSSG, oxidized glutathione; NADH, nicotinamide adenine dinucleotide, reduced form.

[2] Mention of a proprietary product does not imply endorsement by the U. S. Department of Agriculture.

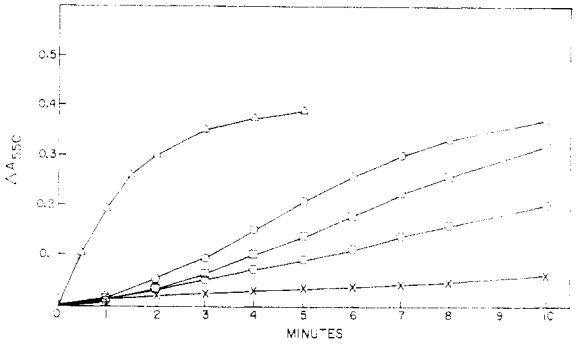


FIGURE 1: Selenite *vs.* GSSG catalysis of reduction of cytochrome *c* by GSH. The reaction cuvets contained 3×10^{-5} M cytochrome *c* and 3.3×10^{-4} M GSH in 0.175 M KCl–0.025 M Tris-Cl buffer (pH 7.45) (basal medium) plus the following additions: none (×); Na_2SeO_3, 10^{-6} M (○); Na_2SeO_3, 5×10^{-6} M (□); Na_2SeO_3, 10^{-5} M (○); or GSSG, 3.3×10^{-3} M (△). The reaction was initiated by the addition of catalyst and was carried out at room temperature. The final reaction volume was 3 ml.

the reduction of cytochrome *c* by GSH. Cystine had no such catalytic effect (data not shown).

Selenium-Catalyzed Reduction of Cytochrome c by Various Sulfhydryl Compounds. A strong catalytic effect of 10^{-5} M selenite on the reduction of cytochrome *c* was still observed if cysteine or mercaptoethylamine was used as the reducing agent in lieu of GSH, but a smaller catalytic effect of Se was obtained when mercaptoethanol or thioglycolate replaced GSH (Table II). Comparison of GSH and cysteine revealed that cysteine was a more effective reductant of cytochrome *c* than GSH both in the absence and in the presence of Se. Dithiothreitol and especially 2,3-dimercaptopropanol were potent reducing agents for cytochrome *c* and even in the absence of Se the latter dithiol caused a very rapid reduction of cytochrome *c*. An acceleration of the rapid reduction rates of cytochrome *c* due to the dithiols was still obtained by the addition of selenite, however.

Inhibition by Cyanide. The reduction of cytochrome *c* by GSH catalyzed by 10^{-5} M selenite could be inhibited 50% by 5×10^{-6} M KCN (Table III). When compared to selenite, selenocyanate was much less active in catalyzing the reduction of cytochrome *c* by glutathione (Table IV).

Effect of Dithiol Inhibitors. The selenite-catalyzed reduction of cytochrome *c* by GSH was only slightly inhibited (10–17%) by 10^{-5} M $HgCl_2$ or $CdCl_2$ and these compounds had to be added at 10^{-3} M to achieve full inhibition (Table V). Arsenite at levels as high as 10^{-3} M had no inhibitory effect on the catalysis of GSH-induced reduction of cytochrome *c* by 10^{-5} M selenite. Even 10^{-2} M arsenite, a concentration 1000 times in excess of the selenite present, inhibited the Se-catalyzed reduction of cytochrome *c* by only 11%.

TABLE I: Effect of Various Selenium Compounds on the Reduction of Cytochrome *c* by GSH.[a]

Compound (Concn, M)	$10^3\Delta A_{550}$ 2 min	5 min	10 min
None	028	043	067
Selenomethionine (10^{-4})	018	040	065
Na_2SeO_4 (10^{-5})	025	044	083
Na_2SeO_4 (10^{-4})	022	073	197
Na_2SeO_3 (10^{-5})	064	227	368
Selenocystine (10^{-5})	207	266	311

[a] The cuvets contained the basal medium (Figure 1) plus the selenium compound at the concentration specified. Reaction conditions are as in Figure 1.

TABLE II: Selenite Catalysis of Reduction of Cytochrome *c* by Various Sulfhydryl Compounds.[a]

Compound	Selenite	$10^3\Delta A_{550}$ 1 min	10 min
Mercaptoethanol	−	053	151
	+	052	232
Thioglycolate	−	034	160
	+	059	258
Mercaptoethylamine	−	019	068
	+	092	400
Cysteine	−	030	170
	+	145	421
Glutathione	−	009	037
	+	007	280
Dithiothreitol	−	064	287
	+	334	346
2,3-Dimercaptopropanol	−	316	
	+	520	

[a] The cuvets contained 3×10^{-5} M cytochrome *c* and 3.3×10^{-4} M of the sulfhydryl compound indicated with or without 10^{-5} M Na_2SeO_3 in the usual KCl–Tris buffer. Reaction conditions are as in Figure 1.

Discussion

The data presented here demonstrate that some selenium compounds can act as effective catalysts for the reduction of cytochrome *c* by glutathione. Feigl and West (1947) were the first to report that certain redox reactions of alkali sulfides could be greatly accelerated by the presence of small amounts of elemental selenium. One of the redox reactions which they investigated was the reduction of Methylene Blue (MB) by sodium sulfide

$$2MB + S^{2-} + 2H_2O \longrightarrow 2HMB + 2OH^- + S^0$$

Elemental selenium was dissolved in the alkali sulfide solution to yield a complex called by them a selenosulfide

$$S^{2-} + Se^0 \longrightarrow [S\cdots Se^0]^{2-}$$

The more reactive selenosulfide ion was considered by Feigl and West to be the catalytically active form of selenium in the Se-catalyzed reduction of Methylene Blue by alkali sulfide

$$2MB + [S\cdots Se^0]^{2-} + 2H_2O \longrightarrow 2HMB + 2OH^- + S^0 + Se^0$$

The elemental selenium formed thereby would be redissolved by the excess alkali sulfide to regenerate the catalytically active selenosulfide ion. The work of Ganther (1971) has shown that glutathione will react with selenite to form the so-called selenotrisulfide derivative of glutathione

$$4GSH + H_2SeO_3 \longrightarrow GSSeSG + GSSG + 3H_2O$$

TABLE III: Effect of Cyanide on Selenite-Catalyzed Reduction of Cytochrome *c* by GSH.[a]

Cyanide Added	$10^3\Delta A_{550}$ (10 min)
None	369
5×10^{-6} M	178
5×10^{-5} M	142
5×10^{-4} M	093

[a] The cuvets contained 3×10^{-5} M cytochrome *c*, 3.3×10^{-4} M GSH, and 10^{-5} M Na_2SeO_3 plus KCN where indicated. Reaction conditions are as in Figure 1.

TABLE IV: Selenite *vs.* Selenocyanate Catalysis of Reduction of Cytochrome *c* by GSH.[a]

Compound (Concn, M)	$10^3\Delta A_{550}$ (20 min)
None	067
KSeCN (10^{-4})	092
KSeCN (10^{-3})	228
Na_2SeO_3 (10^{-5})	405

[a] The cuvets contained the basal medium (Figure 1) plus the compound indicated. Reaction conditions are as in Figure 1.

In the presence of excess GSH and at physiological pH the initial selenotrisulfide product reacts further to yield the glutathione selenopersulfide

$$GSSeSG + GSH \longrightarrow GSSeH + GSSG$$

Massey *et al.* (1971) have suggested that the catalytic effect of certain commercial samples of oxidized glutathione on the reduction of cytochrome *c* by GSH is due to the presence of glutathione trisulfide (GSSSG) as an impurity. The catalytically active species involved in the rapid reduction of cytochrome *c* was thought to be the persulfide, GSS^-, and the following mechanism was proposed for the catalytic effect of GSSSG on cytochrome *c* reduction by GSH

$$GSSSG + GS^- \rightleftharpoons GSS^- + GSSG$$

$$2GSS^- + 2\text{cyt } c^{3+} \longrightarrow 2GSS\cdot + 2\text{cyt } c^{2+}$$

$$2GSS\cdot \rightleftharpoons GSSSSG$$

$$GSSSSG + GS^- \rightleftharpoons GSSSG + GSS^-$$

$$\text{Net:} \quad 2GS^- + 2\text{cyt } c^{3+} \longrightarrow GSSG + 2\text{cyt } c^{2+}$$

An analogous series of reactions could be written to account for the catalytic effect of selenium on the reduction of cytochrome *c* by GSH

$$GSSeSG + GS^- \rightleftharpoons GSSe^- + GSSG$$

$$2GSSe^- + 2\text{cyt } c^{3+} \longrightarrow 2GSSe\cdot + 2\text{cyt } c^{2+}$$

$$2GSSe\cdot \rightleftharpoons GSSeSeSG$$

$$GSSeSeSG + GS^- \rightleftharpoons GSSeSG + GSSe^-$$

$$\text{Net:} \quad 2GS^- + 2\text{cyt } c^{3+} \longrightarrow GSSG + 2\text{cyt } c^{2+}$$

Massey *et al.* (1971) found that the catalytic activity of certain samples of GSSG on the reduction of cytochrome *c* by GSH was lost on incubation with KCN. This was thought to be due to the destruction of the catalytically active impurity, glutathione trisulfide (GSSSG)

$$GSSSG + CN^- \longrightarrow GSSG + SCN^-$$

The inhibitory effect of cyanide on the selenium-catalyzed reduction of cytochrome *c* by GSH reported here could be accounted for by a similar reaction

$$GSSeSG + CN^- \longrightarrow GSSG + SeCN^-$$

This possibility seems reasonable especially in light of the fact that the selenocyanate ion itself was shown to have very little catalytic effect on the reduction of cytochrome *c* by GSH.

The reactions outlined above could also explain the differences seen here in the catalytic potency of the various forms of Se in accelerating the reduction of cytochrome *c* by glutathione. For example, the slower catalysis seen with selenate might occur because selenate would first have to be reduced to selenite before it could react with GSH to form the catalytically active selenopersulfide. On the other hand, the very rapid catalytic activity of selenocystine would presumably be due to the fact that the selenium in this compound already exists in the selenide valence state. This would allow for the generation of selenocysteine

$$GS^- + CysSeSeCys \longrightarrow CysSe^- + GSSeCys$$

which might then reduce the cytochrome *c* directly. Although selenium is also at the selenide valence level in the catalytically inactive selenomethionine, the inactivity of this selenium derivative is probably due to the fact that the selenium in the molecule is masked by two relatively inert selenium–carbon bonds.

We have been able to confirm the results of Massey *et al.* (1971) that different commercial samples of GSSG show dif-

TABLE V: Effect of Dithiol Inhibitors on Selenite-Catalyzed Reduction of Cytochrome *c* by GSH.

Compound (Concn, M)	% Inhibition[a]
$HgCl_2$ (10^{-5})	17
$HgCl_2$ (10^{-4})	72
$HgCl_2$ (10^{-3})	96
$CdCl_2$ (10^{-5})	10
$CdCl_2$ (10^{-4})	47
$CdCl_2$ (10^{-3})	98
$NaAsO_2$ (10^{-5})	0
$NaAsO_2$ (10^{-4})	0
$NaAsO_2$ (10^{-3})	0
$NaAsO_2$ (10^{-2})	11

[a] Represents 1 − (Δabsorbance$_{550}$ (with inhibitor)/Δabsorbance$_{550}$ (without inhibitor))100. The cuvets contained the basal medium (Figure 1) plus 10^{-5} M Na_2SeO_3 and the inhibitor at the concentration specified. The reaction time was 10 min. Reaction conditions are as in Figure 1.

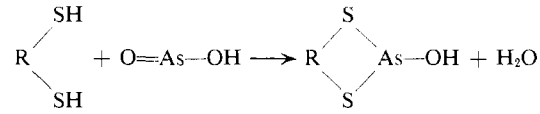
ferent activities in catalyzing the reduction of cytochrome *c* by GSH. Whether the catalytic activity of some GSSG samples is due to selenium contaminants is not known at present, but the possibility of GSSeSG or other selenium impurities should not be ruled out. Substantial variation in the content of trace mineral contaminants in commercial samples of glutathione has been reported (Cash and Gardy, 1965). It should also be pointed out that different commercial samples of GSSG have different potencies as mitochondrial swelling agents (Levander *et al.*, 1972) and that those samples of GSSG which are most effective as swelling agents are those which are also generally most effective as catalysts in the reduction of cytochrome *c* by GSH.

A comparison between the ability of Se to catalyze the reduction of cytochrome *c* by GSH seen here and the ability of Se to accelerate the mitochondrial swelling induced by GSH described earlier (Levander *et al.*, 1973) might be helpful in explaining the latter phenomenon. First of all, both Se and certain samples of GSSG are effective catalysts for either process. But the fact that selenite was the most effective catalyst for mitochondrial swelling induced by GSH, whereas selenocystine was the best catalyst for cytochrome *c* reduction by GSH, might indicate that the swelling process is most effectively caused by a selenopersulfide, whereas cytochrome *c* can be readily reduced by a compound containing a selenol group such as selenocysteine. Of course, differences in the permeability of the mitochondria to different Se derivatives might also play a role here. The observation that selenomethionine had no catalytic effect on the reduction of cytochrome *c* by GSH but nonetheless still had some activity in promoting mitochondrial swelling induced by GSH suggests that mitochondria may be able to metabolize this compound to a catalytically active form of Se.

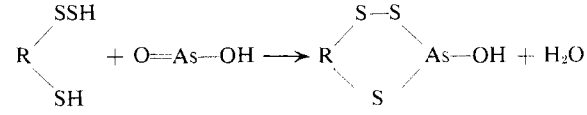
Generally, a good correlation was observed between the ability of a thiol to cause mitochondrial swelling and its ability to reduce cytochrome *c*. For example, cysteine was shown to be the most potent compound in causing both processes, whereas mercaptoethanol and thioglycolate were the least potent. However, a major discrepancy between the mitochondrial swelling results and the cytochrome *c* reduction results was seen when dithiothreitol and 2,3-dimercaptopropanol were studied. Although both of these dithiols were very active reducing agents for cytochrome *c* even in the absence of Se, neither compound had activity in accelerating mitochondrial swelling even in the presence of Se. This lack of effect in swelling could be due to an inhibition of respiration by these compounds, since there is a dithiol-sensitive factor in the respiratory chain (Slater, 1949) and thiol-mediated swelling appears to depend on respiration (Lehninger and Schneider, 1959).

Other discrepancies between the mitochondrial swelling results and the cytochrome *c* reduction results were revealed in the experiments with dithiol inhibitors. For example, Hg^{2+} and Cd^{2+} at 10^{-5} M almost totally inhibited GSH plus selenite mitochondrial swelling, whereas the same concentrations of these ions scarcely inhibited GSH plus selenite reduction of cytochrome *c*. These results seemed to rule out the possibility of a direct reaction between the heavy metals and the Se catalyst since the selenite was present at the same concentration (10^{-5} M) in both test systems and one might expect to observe a similar inhibition in both cases if only a heavy metal complexing of Se were involved. Furthermore, in the studies with mitochondria one would expect a large amount of nonspecific binding of the heavy metals by the subcellular particles which would reduce the effective concentration of metal ion available to complex with Se. Since the heavy metals cannot effectively tie up all the GSH which is present in 30× excess in the incubation medium, they may inhibit GSH plus selenite swelling by reacting with the mitochondrial site at which Se acts. This idea is strengthened by the experiments with arsenite which showed that arsenite had very little inhibitory activity against the selenite-catalyzed reduction of cytochrome *c* by GSH even at a concentration 1000× greater than that which strongly inhibited GSH plus selenite mitochondrial swelling.

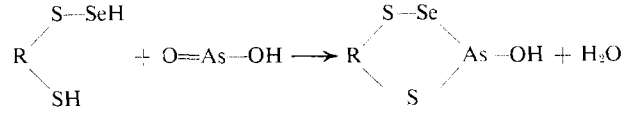
The inhibitory effects of the heavy metals and arsenite on GSH plus selenite mitochondrial swelling imply that a vicinal dithiol is involved in this process since these inhibitors are known to complex with such groups



Although blockage of vicinal dithiols represents the best known mechanism of arsenite inhibition, Massey and Edmondson (1970) have postulated that arsenite inhibits xanthine oxidase by forming a complex with a persulfide and a neighboring sulfhydryl group



By analogy, one could postulate an inhibitory complex of arsenite with a selenopersulfide and a neighboring sulfhydryl group



The role of Se in catalyzing mitochondrial swelling due to GSH might therefore involve a dithiol necessary for the reduction of cytochrome *c in situ*. Slater (1949) has described a 2,3-dimercaptopropanol-sensitive factor which is necessary for the transfer of electrons from cytochrome *b* to cytochrome *c*. Important evidence in support of the concept that Se may play a role in electron transport derives from the recent work of Turner and Stadtman (1973) who found that a component of the electron transfer chain of clostridial glycine reductase is a selenoprotein. Moreover, Diplock (1973) has postulated a possible role for Se in the electron transfer system of rat liver microsomes.

Although a role for Se in electron transport would seem to provide a reasonable explanation for the effect of Se seen in GSH-induced mitochondrial swelling, one should not disregard other possible sites of action of Se in mitochondrial metabolism. The most thoroughly documented role for a vicinal dithiol in mitochondrial metabolism is that of lipoic acid which is considered to be the locus of the inhibitory effect of arsenite in the pyruvate and α-ketoglutarate dehydrogenase complexes (Sanadi *et al.*, 1959). Dihydrolipoyl dehydrogenase, in fact, was suggested as the probable site of the enzymatic breakdown of respiration in vitamin E and Se-deficient liver homogenates (Schwarz *et al.*, 1962). A breakdown at this point would explain the fact that the defect in respiration in vitamin E and Se-deficient liver preparations is observed more

consistently when NADH-linked substrates are used than when succinate is used (Grove *et al.*, 1965). It should also be pointed out that the phenomenon of respiratory decline can be induced in mitochondria by the addition *in vitro* of traces of Cd^{2+} or arsenite (Corwin and Schwarz, 1963). Since there was a partial blockage of GSH plus selenite swelling due to amytal or antimycin A, it seems possible that Se could exert some influence at the dehydrogenase level.

In addition to possible effects of Se at the electron transport or dehydrogenase level, still another aspect of mitochondrial function may be influenced by Se, namely oxidative phosphorylation. Fluharty and Sanadi (1960) have presented evidence for a vicinal dithiol in oxidative phosphorylation and Painter and Hunter (1970) have considered the GSSG-catalyzed reduction of cytochrome *c* by GSH as a possible model for oxidative phosphorylation. As discussed above, Massey *et al.* (1971) showed that the active species involved in the rapid GSH reduction of cytochrome *c* catalyzed by GSSG is probably the persulfide, GSS^-. Massey and colleagues suggested therefore that a persulfide or related structure might be involved in the process of oxidative phosphorylation. On the basis of the work reported here, a selenopersulfide could be considered the related structure in question.

One final comment regarding possible sites of action of Se in mitochondrial function concerns the fact that several Se-containing homologs of nonheme iron proteins have been prepared by substituting Se for the labile S in the molecule (Fee and Palmer, 1971; Münck *et al.*, 1972; Mukai *et al.*, 1973). A hypothesis has been advanced which states that the primary function of vitamin E as an antioxidant in biological systems is not to protect polyunsaturated lipids but rather is to prevent autoxidation of selenide which might occur in nonheme iron proteins (Diplock *et al.*, 1971). Whether any of the effects of Se discussed above involve a selenononheme iron protein cannot be ascertained at present.

Added in Proof

Since submission of this manuscript, Whanger *et al.* (1973) have described a selenium-containing protein from lamb muscle which contains a heme group identical to that of cytochrome *c*.

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