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# Oxidation of 3,3′-Diaminobenzidine by Rat Liver Mitochondria†

Wendy Cammer* and Cyril L. Moore

ABSTRACT: The dye 3,3′-diaminobenzidine (DAB) has been used previously (Seligman, A. M., Karnovsky, M. J., Wasserkrug, H. L., and Hanker, J. S., *J. Cell Biol. 38*, 1 (1968)) for ultrastructural location of cytochrome oxidase in liver and kidney mitochondria. An *in vitro* study of the biochemical site of DAB oxidation by liver mitochondria is reported here. Studies of mitochondrial oxygen utilization, using the optimal DAB concentration of 4 mM in a highly buffered medium, showed that DAB oxidation was not inhibited by antimycin A but was inhibited by cyanide. Mitochondria depleted of cytochrome *c* required addition of exogenous cytochrome *c* in order to oxidize DAB, and polylysine inhibited mitochondrial oxidation of DAB. These studies show that the site of interaction of DAB with the respiratory chain is at cytochrome *c* and therefore support the conclusion that the ultrastructural location of cytochrome *c* is at the outer surface of the inner mitochondrial membrane. Reduction of cytochrome *c* by DAB was demonstrated spectrophotometrically and indicated a reduction of three to four cytochrome *c* molecules by each DAB molecule. Mitochondrial oxidation of DAB gave a stoichiometry of one DAB molecule oxidized by each oxygen molecule. Production of free radicals during oxidation of DAB was demonstrated using the sulfite chain reaction. Based on these data, a mechanism is proposed where DAB loses four electrons to yield a free radical intermediate, which subsequently polymerizes to give the final product.

Seligman *et al.* (1968) have reported cytological evidence that the product of mitochondrial oxidation of the dye DAB[1] accumulates on the outer surface of the mitochondrial inner membrane. They concluded that the reaction of electron donors with cytochrome *c* was located at that intramitochondrial site. Because such information is important in understanding enzyme compartmentation in mitochondria, a thorough *in vitro* study of the biochemical steps involved in DAB oxidation by rat liver mitochondria is being reported here.

## Materials and Methods

DAB was obtained as the tetrachloride from the Sigma Chemical Co. and was dissolved in 10 mM Tris-chloride (pH 7.4). TMPD was obtained as the dichloride from Eastman Organic Chemicals. Sodium sulfite was obtained from Merck and Co. and sodium hydrosulfite was obtained from Baker Chemical Co. Other reagents and substrates were obtained from the Sigma Chemical Co. Stock solutions of glutamic, malic, and succinic acids were titrated to pH 7.4 using Trizma base, and NaADP was kept at pH 6.9. Polylysine hydrobromide (Sigma Types VI-B and VII-B) was dissolved in the respiration medium described below.

Rat liver mitochondria were prepared by the usual method (Johnson and Lardy, 1967) of homogenization and differential

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[1] Abbreviations used are: DAB, 3,3′-diaminobenzidine; TMPD, tetramethylphenylenediamine; ADP, adenosine 5′-diphosphate.

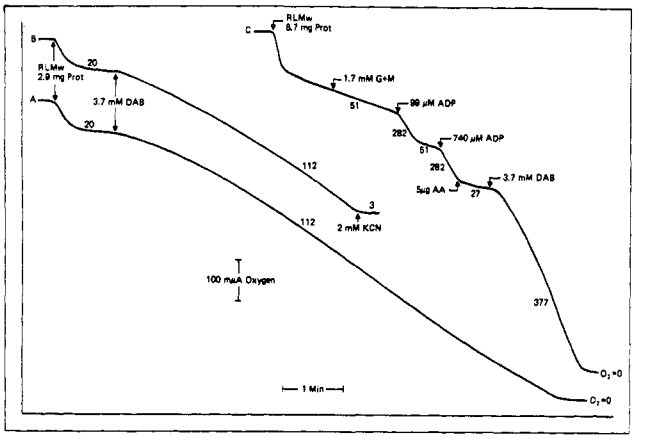



FIGURE 1: Effect of the respiratory inhibitors antimycin A and KCN on mitochondrial oxidation of DAB. Mitochondria and reagents were added to respiration medium in the 1.7-ml oxygen electrode chamber, as shown in the traces. The numbers alongside the traces are respiration rates in millimicroatoms of oxygen per minute.

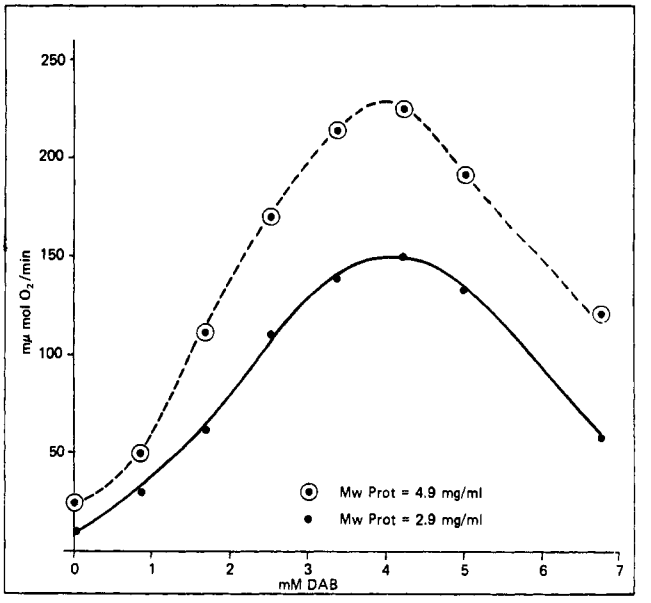



FIGURE 2: The effect of DAB concentration on the rate of mitochrondrial respiration. In order to obtain each point, mitochondria were added to respiration medium in the oxygen electrode chamber to give the final protein concentration shown, in a total volume of 1.7 ml. One minute later DAB was added to give the desired final concentration. Rates of oxygen uptake are plotted in millimicromoles of oxygen per minute (nmol of $O_2$/min). Respiration was allowed to proceed until the oxygen in the chamber was exhausted, and the most rapid rate of respiration was measured.

centrifugation. The solution in which the minced rat liver was homogenized contained 0.25 M sucrose, 0.5 mM EDTA, and 5 mM Tris-chloride (pH 7.4). The mitochondria were washed two times with ST (0.25 M sucrose plus 5 mM Tris-chloride, pH 7.4) and were resuspended in ST.

Respiration was measured polarographically (Chappell, 1961) using the Clark oxygen electrode in a 1.7-ml water-jacketed oxygen electrode chamber. Respiration medium consisted of 0.25 M sucrose, 15 mM Tris-chloride (pH 7.4), 60 mM sodium phosphate (pH 7.4), 20 mM KCl, and 5 mM $MgCl_2$. The high concentration of phosphate buffer was necessary to prevent acidification of the medium on addition of the DAB tetrachloride solution. The DAB tetrachloride was not titrated before use because the free base tended to precipitate in the concentrated stock solution. Mitochondria and reagents were added as described in the figures and text.

A Gilford spectrophotometer was used for measurements of cytochrome *c* reduction in the absence of mitochondria. The measuring wavelength was 551 mμ, and the reference wavelength was 540 mμ. Reduced minus oxidized difference spectra of mitochondrial suspensions were obtained using a Phoenix precision recording spectrophotometer.

In carrying out temperature control experiments the respiration medium (volume 1.7 ml) in the water-jacketed oxygen electrode chamber was allowed to reach a steady temperature prior to addition of mitochondria. A Haake circulating bath was used to maintain the medium temperature. After a 1-min equilibration time, DAB was added to a final concentration of 3.3 mM, and the respiration was monitored. The maximal rate of oxygen uptake was used for the calculation. A comparative study was made with 135 μM TMPD plus 670 μM ascorbate as substrate in place of DAB.

Cytochrome *c* depleted mitochondrial were prepared by the method of Lenaz and MacLennan (1967), and the absence of cytochrome *c* was confirmed using the Phoenix precision spectrophotometer.

Protein determinations were made according to the method of Lowry *et al.* (1951).

## Results

In Figure 1 trace A shows DAB-supported respiration in isolated mitochondria. Because it had been postulated (Seligman *et al.*, 1968) that DAB was oxidized *via* the cytochrome *c*

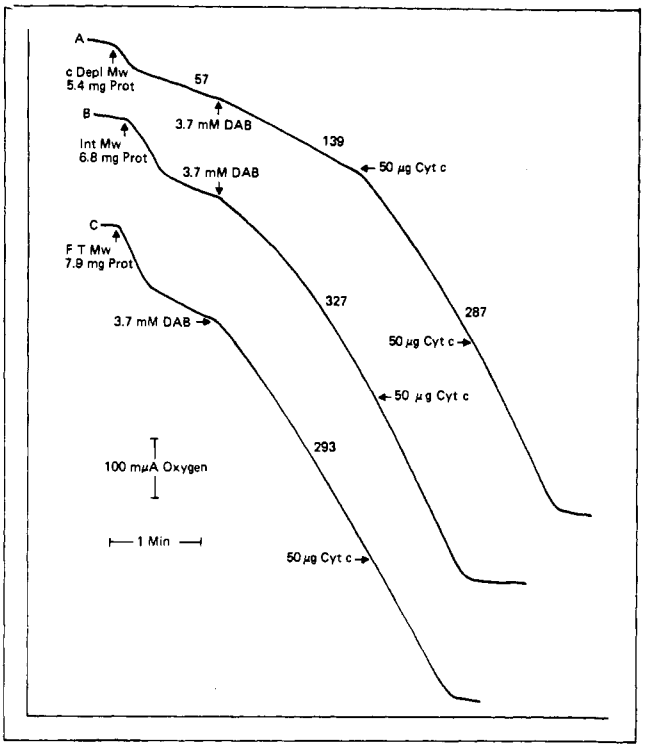
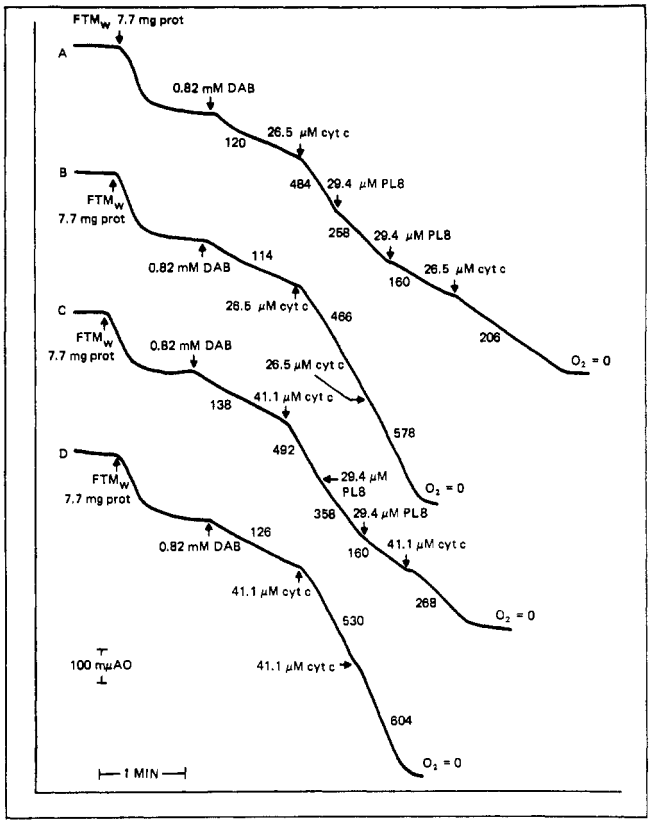



FIGURE 3: Oxidation of DAB by intact (Int), frozen and thawed (FT), and cytochrome *c* depleted (c Depl) mitochondria. Mitochondrial preparations and reagents were added to respiration medium in the 1.7-ml oxygen electrode chamber, as shown in the traces. The numbers next to the traces are respiration rates in millimicroatoms of oxygen per minute.



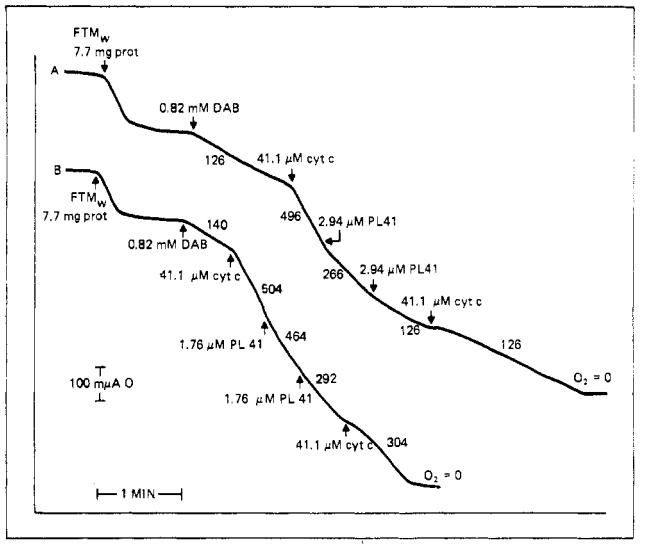




FIGURE 4: Effect of polylysine on DAB oxidation. Rat liver mitochondria (washed) which had been frozen approximately 15 hr and then thawed (FT Mw), DAB, and cytochrome *c* were added to respiration medium, as shown, in the 1.7-ml oxygen electrode chamber. Stock solutions of polylysine of molecular weight approximately 8000 (PL 8) or 41,000 (PL 41) were prepared in respiration medium, and aliquots were added in the figures. The numbers next to the traces are respiration rates in millimicroatoms of oxygen per minute.

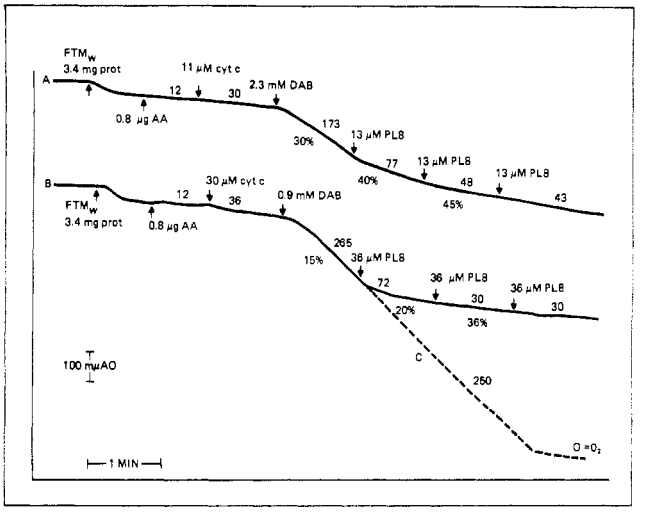



FIGURE 5: Steady-state levels of cytochrome *c* reduction during polylysine inhibited oxidation of DAB by frozen and thawed rat liver mitochondria. Mitochondria which had been frozen approximately 15 hr and then thawed (FTMw) and reagents were added, as shown, to respiration medium in the 1.7-ml oxygen electrode chamber. The per cent reduction of cytochrome *c* is shown below portions of the traces, with dithionite reduced cytochrome *c* minus oxidized cytochrome *c* taken as 100% reduction. Antimycin A was included in the system to prevent electron transport from endogenous substrates to cytochrome *c*. In order to determine the extents of cytochrome *c* reduction, a mixture of mitochondria, antimycin A, and cytochrome *c*, in the same concentrations as used in the polarographic determinations, was divided between two cuvets, and a base line of light absorption was recorded. Then dithionite was added to the experimental cuvet and the spectrum was scanned in order to obtain 100% cytochrome *c* reduction. Then DAB was added to the reference cuvet, and the extent of decrease in the cytochrome *c* absorption was taken as per cent reduction by DAB alone. These determinations were repeated, adding the appropriate concentrations of polylysine to the experimental cuvet before DAB. The numbers above the traces are respiration rates in millimicroatoms of oxygen per minute.

to cytochrome oxidase portion of the respiratory chain, antimycin A, which is known to inhibit electron transport between cytochromes *b* and *c* (Chance and Williams, 1956), was used as shown in trace C. With the $NAD^+$-linked substrates, glutamate plus malate, ADP produced a rapid rate of respiration (state 3), which declined after the ADP had been phosphorylated (state 4). Subsequently sufficient ADP to exhaust all the oxygen in the system caused rapid respiration which was inhibited by 5 μg of antimycin A. However, added DAB was oxidized at a rate of 377 natoms of O/min. DAB oxidation was inhibited by cyanide (trace B) as compared to the control (trace A).

Figure 2 shows the effect of DAB concentration on mitochondrial respiration. Final concentrations of $DAB(Cl^-)_4$ greater than 4.2 mM in the reaction medium lowered the pH to below 7.0, and were inhibitory, thereby preventing a true optimum rate of oxidation of DAB.

In order to demonstate the requirement for cytochrome *c* in DAB oxidation, cytochrome *c* depleted mitochondria were used. The data in Figure 3, trace A, indicate that the depleted mitochondria had only enough residual cytochrome *c* to increase the rate of respiration from 57 to 139 natoms of O per min on addition of DAB. Addition of 50 μg of cytochrome *c* increased the rate to 287 natoms of O/min. Addition of another aliquot of cytochrome *c* gave no further stimulation of respiration. With intact mitochondria (trace B), DAB oxidation was preceded by an initial lag, possibly attributable to problems of permeability. This respiration is insensitive to added cytochrome *c*. In contrast, when frozen and thawed mitochondria obtained after the first step in the preparation of the cytochrome *c* deficient mitochondria (Lenaz and MacLennan, 1967) were used, there was no lag in DAB oxidation, as shown in trace C, and no stimulation by cytochrome *c*. While insensitivity to cytochrome *c* is usual for intact mitochondria utilizing high concentrations of DAB, at lower DAB concen-

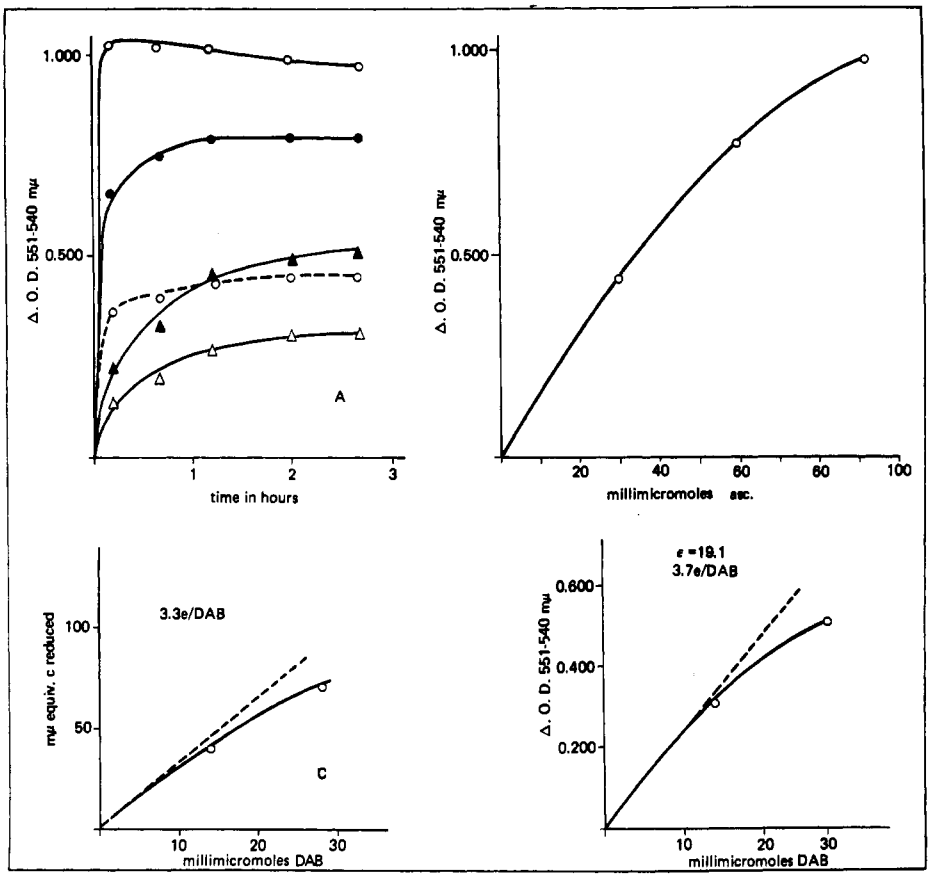



FIGURE 6: Stoichiometry of reduction of cytochrome *c* by DAB. In order to obtain the graph on the upper left side, 13.9 (open triangles) or 27.8 (solid triangles) nmol of DAB was added to 300 nmol of cytochrome *c* in 3 ml of 60 mM sodium phosphate buffer (pH 7.4). The absorbances at 541 and 550 mμ were measured in the Gilford spectrophotometer over a 3-hr period. A cytochrome *c* blank and cytochrome *c* plus 30 (open circles, dotted line), 60 (closed circles), or 90 (open circles, solid line) nmol of ascorbic acid were measured similarly, to be used as standards. The final absorbances from the ascorbate curves are plotted on the upper right side to give a standard curve. Assuming a reduction of two cytochrome *c* molecules by each ascorbate molecule, a reduction of 3.3 cytochrome *c* molecules by each DAB molecule is obtained in the graph on the lower left side. On the lower right side a similar result (3.7e) can be obtained directly using the extinction coefficient of 19.2 $cm^{-1}$ $mM^{-1}$ for cytochrome *c* at 551 minus 540 mμ.

trations the respiration of intact mitochondria is stimulated by cytochrome *c*.

The oxidation of cytochrome *c* by cytochrome oxidase has been shown to be inhibited by large cationic molecules (Person and Fine, 1961) including cytochrome *c* (Smith and Conrad, 1956), which is itself a basic protein. Mitochondria frozen approximately 15 hr become permeable to cytochrome *c* and the basic polypeptide polylysine. In trace A of Figure 4A addition of cytochrome *c* to frozen and thawed mitochondria oxidizing DAB increased the rate of respiration from 120 to 484 natoms of O per min. Addition of polylysine of molecular weight approximately 8000 (PL8) produced a slower rate of respiration, and a second addition of polylysine gave further inhibition. A subsequent addition of cytochrome *c* produced a slight increase in the rate of respiration. Trace B shows the rates of respiration utilizing DAB and the two cytochrome *c* concentrations shown in trace A. Using these data, 466 and 578 natoms of O per min, as controls, the inhibitions of respiration before and after the final addition of cytochrome *c* in trace A can be calculated as 66 and 64%, respectively. Traces C and D show a similar experiment, using somewhat higher cytochrome *c* concentrations. The inhibition of respiration before and after the final addition of cytochrome *c* in trace C can be calculated as 70 and 56%, respectively. These figures show that additional cytochrome *c* was able partially to overcome the polylysine inhibition. Addition of polylysine before or after cytochrome *c* and DAB resulted in the same degree of inhibition.

Figure 4B shows the effect of a higher molecular weight polylysine in the same system. Although this polylysine was of molecular weight 41,000, or approximately five times PL8, one-tenth the concentration was capable of producing an inhibitor effect which could not be overcome by further addition of cytochrome *c*.

Since it has been reported that basic proteins inhibit the oxidation of reduced cytochrome *c* (Smith and Conrad, 1961), it was of interest to determine whether the polylysine was inhibiting reduction of cytochrome *c* by DAB or reoxidation of cytochrome *c* by cytochrome oxidase. Spectrophotometric data (551–540 mμ) on cytochrome *c* reduction in the absence of mitochrondria indicated that polylysine did not inhibit the reduction of cytochrome *c* by DAB. The concentrations of cytochrome *c*, polylysine, and DAB used were equal to those used in obtaining the data in Figure 4A. In order to obtain a more realistic assessment of the site of polylysine inhibition, the steady-state levels of reduced cytochrome *c* (per cent reduction) shown below the polarographic traces in Figure 5 were obtained from reduced minus oxidized difference spectra, using identical reagents and mitochondrial concentrations in the cuvettes as in the oxygen electrode chamber. Antimycin A was included in the system to prevent electron transport from endogenous substrate to cytochrome *c*. These data show that

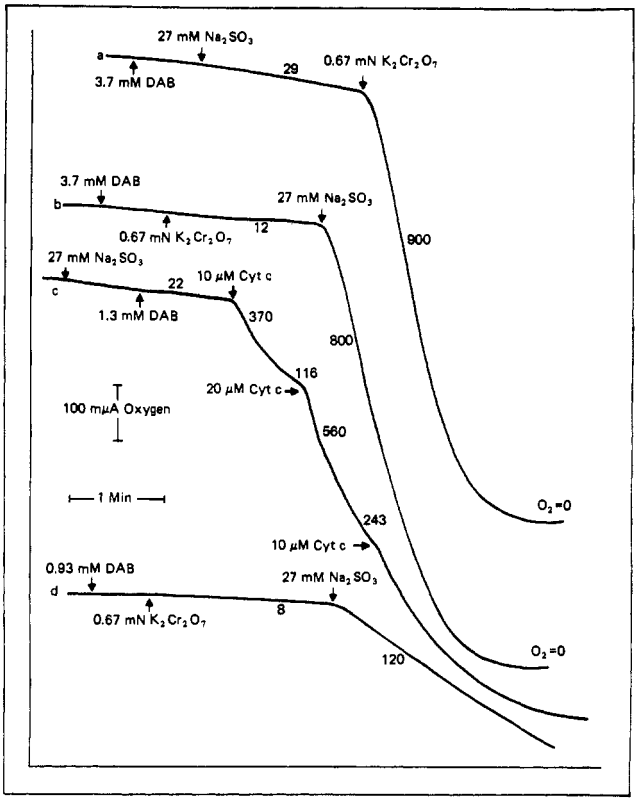



FIGURE 7: Initiation of the chain reaction for sodium sulfite oxidation by oxygen during DAB oxidation by cytochrome *c* (trace c) or potassium dichromate (traces a, b, and d). Reagents were added as shown to 1.7 ml of respiration medium in the oxygen electrode chamber. The numbers next to the traces are rates of oxygen utilization in millimicroatoms of oxygen per minute.

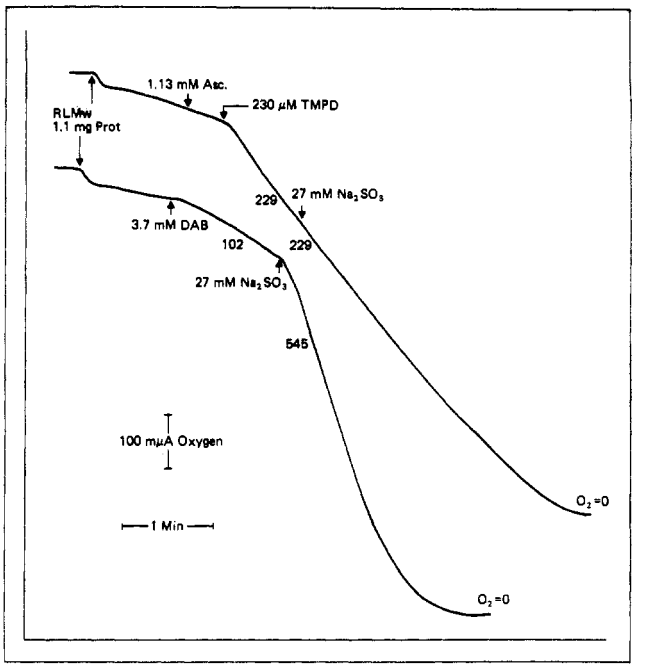



FIGURE 8: The effect of mitochondrial oxidation of DAB or ascorbate plus TMPD on the chain reaction of sodium sulfite with oxygen. Mitochondria and reagents were added as shown to respiration medium in the 1.7-ml oxygen electrode chamber. The numbers next to the traces are rates of oxygen utilization in millimicroatoms of oxygen per minute.

polylysine inhibited reoxidation of the cytochrome *c* by cytochrome oxidase, after the cytochrome *c* was reduced by DAB. In trace B a higher proportion of cytochrome *c* was in the oxidized form under all conditions, as compared to trace A, although the rate of respiration with DAB was higher than in trace A. The higher proportion of oxidized cytochrome was observed because a larger amount of cytochrome *c* was added in trace B, and most of the added cytochrome was in the oxidized form. The slight increases in respiration after the initial additions of cytochrome *c* show that some of the added cytochrome was, however, in the reduced state. These results tend to confirm previous reports of polyion inhibition of oxidation of cytochrome *c* by cytochrome oxidase and are consistent with cytochrome *c* mediation of electron transport between DAB and cytochrome oxidase.

The reduction of cytochrome *c* by two different concentrations of DAB was carried out in the absence of mitochondria, using the Gilford spectrophotometer at 551 and 540 mμ. The two upper plots in Figure 6 show, respectively, the time course of reduction of cytochrome *c* by DAB and ascorbate and the use of ascorbate to standardize measurement of cytochrome *c* reduction. Assuming a reduction of two cytochrome *c* molecules by each ascorbate molecule, a reduction of 3.3 cytochrome *c* molecules by each DAB molecule is obtained in the graph on the lower left side. On the lower right side a similar result (3.7e) can be obtained directly using the extinction coefficient of 19.2 $cm^{-1}$ $mM^{-1}$ for cytochrome *c* at 551 − 540 mμ.

A chain reaction involving sodium sulfite and oxygen is initiated in the presence of free radicals, and results in an accelerated reduction of oxygen (Fridovich and Handler, 1960). Oxygen utilization for the sulfite reaction concomitant with DAB oxidation is shown in Figure 7. In Figure 7, Trace C, two sequential additions of cytochrome *c* to respiration medium containing sulfite and DAB in the oxygen electrode chamber in the absence of mitochondria initiated oxygen utilization at rates of 370 and 560 natoms of O per min, respectively. Trace A shows rapid utilization of oxygen when dichromate was added as oxidant for DAB in the presence of sulfite. Trace B shows the requirement of sulfite for oxygen uptake. As shown in trace D, the rate of oxygen uptake is a function of the DAB concentration. This is possibly due to the rate-limiting level of free-radical initiators of the chain reaction.

Although previous authors assumed that only $O_2\cdot^-$ free radicals were capable of initiating the sulfite chain reaction (Fridovich and Handler, 1960), the results of Figure 7, where nonenzymatic oxidation of DAB by dichromate initiated sulfite oxidation, suggest that DAB free radicals are capable of generating either $SO_3\cdot^-$ or $O_2\cdot^-$ radicals directly.

In Figure 8 the lower trace shows that mitochondrial oxidation of DAB also initiated the chain reaction for oxidation of sulfite by oxygen. The upper trace shows that mitochondrial oxidation of TMPD plus ascorbate, which have been shown to be oxidized by the cytochrome *c* to cytochrome oxidase portion of the respiratory chain (Tyler *et al.*, 1966), did not initiate the sulfite reaction, suggesting that TMPD free radicals either are not generated in ascorbate oxidation (*cf.* Park *et al.*, 1957) or, if generated, are too low in concentration to be capable of initiating an intermediate in the sulfite chain reaction. In this experiment (Figure 8) a slightly suboptimal amount of DAB was used, and the sulfite oxidation reaction was initiated before DAB oxidation reached its maximum rate. If oxygen free radicals are present (Fridovich and Handler, 1961), their location and possible low concentration inside

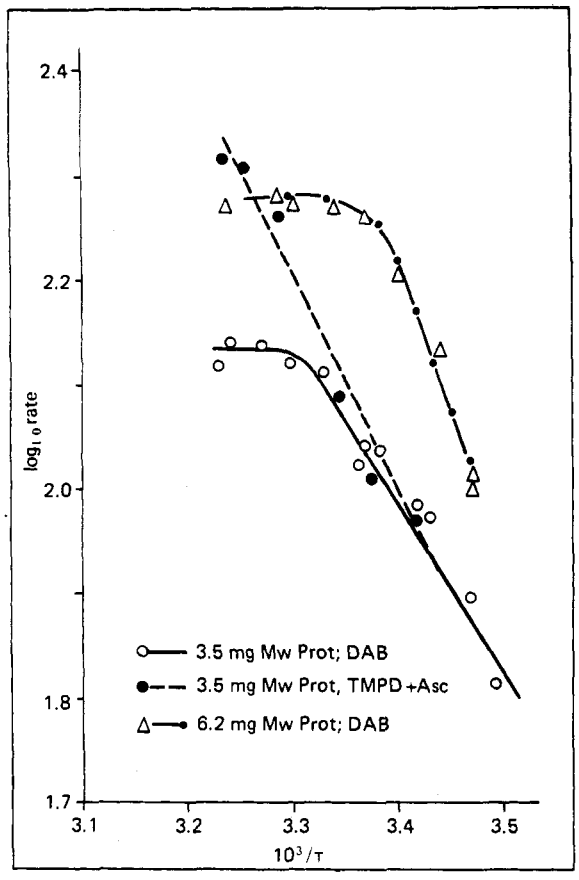
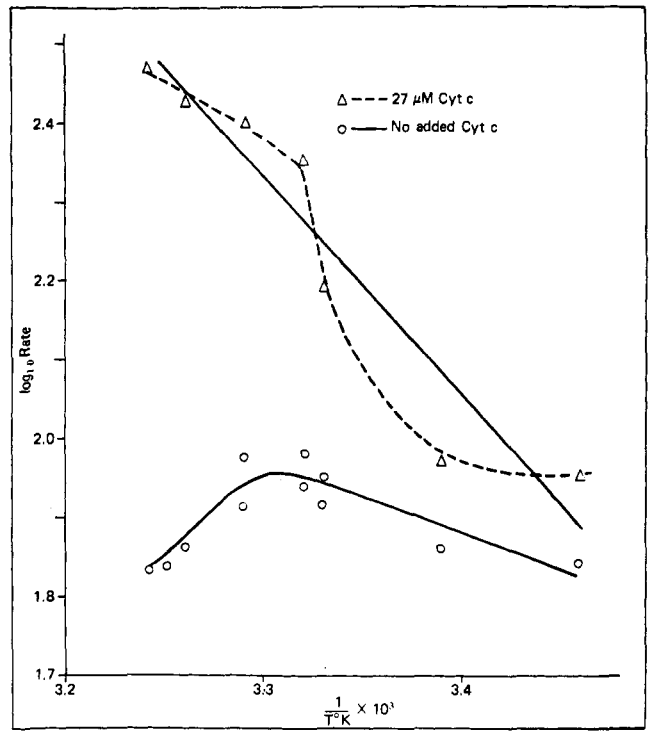



FIGURE 9: The effect of temperature on mitochondrial DAB oxidation. The oxygen electrode (1.7-ml chamber) and Haake circulating bath were used as described in Materials and Methods. DAB (3.3 mM) or ascorbate (670 μM) plus TMPD (135 μM) was used as substrate for 3.5 mg of mitochondrial protein, and DAB was used with 6.2 mg of mitochondrial protein as well. After a steady temperature was obtained, mitochondria were added to respiration medium (STRM) in the oxygen electrode chamber. One minute later DAB or TMPD plus ascorbate was added. The reaction was allowed to proceed until the oxygen in the chamber was exhausted, and the maximum rate of respiration was measured. The logarithm of the respiration rate is plotted against the reciprocal of the temperature in degrees Kelvin.




FIUURE 10: The effect of temperature on mitochondrial DAB oxidation in the presence and absence of added cytochrome *c*. The procedure was similar to the method described for Figure 10, except that 27 μM cytochrome c was added to the medium where indicated, 30 sec before addition of DAB. A different batch of mitochondria was used, with 3.0 mg of protein in the oxygen electrode chamber. The logarithm of the rate of respiration is plotted against the reciprocal of the temperature in degrees Kelvin. For the trace with cytochrome *c* present (triangles), the dotted line shows an alternative interpretation of the data.

intact mitochondria must render them inaccessible to sulfite ions, in the case of TMPD oxidation.

Further investigation of the reaction mechanism involved polarographic measurement of the rates of DAB oxidation at various temperatures. The Arrhenius plot (Figure 9) shows that when DAB was the substrate, the respiratory rate increased with temperature, levelling off around 30°. With TMPD plus ascorbate, however, the rate continued to increase with temperature beyond this point. Since the reduction of cytochrome *c* by DAB might be the rate limiting step for respiration above 30°, mitochondrial oxidation of DAB was studied as a function of temperature in the presence and absence of added cytochrome *c*. Figure 10 shows that with this mitochondrial preparation there was a requirement for added cytochrome *c* for a maximum rate of DAB oxidation at all the temperatures tested. The cytochrome *c* stimulated rate increased with increasing temperature up to the highest temperature tested (36°). However, above 31° the rate of DAB oxidation in the absence of exogenous cytochrome *c* decreased with increasing temperature. This decrease in rate is possibly due to dissociation of some of the intramitochondrial cytochrome *c* from the respiratory chain. The oxidation of ascorbate, however, would be taking place at optimal TMPD concentrations and would therefore be less sensitive to cytochrome *c* dissociation. When excess cytochrome *c* was made freely available to the electron transport system of mitochondria, thus avoiding the possible artifact of dissociation of endogenous cytochrome *c*, the true rate of DAB oxidation as a function of temperature could be evaluated.

Mitochondria which had been frozen and thawed to render them highly permeable to cytochrome *c* were used in obtaining Figure 11, and an excessive amount of cytochrome *c* (71 μM) was added to the respiration medium. It was now possible to oxidize small aliquots of DAB rapidly and, in effect, to titrate oxygen with DAB. Rat liver mitochondria which had been frozen and thawed, catalase, and cytochrome *c* were added to respiration medium in the oxygen electrode chamber at 34.5°, and 70 nmol of DAB were added (top trace), resulting in a burst of rapid respiration. When respiration had become slow again, presumably because all the DAB had been oxidized, an aliquot of DPNH was added to check the calibration of the system. Addition of more DPNH exhausted the available oxygen. The lower trace shows a similar experiment, using 139 nmol of DAB. Measurement of the amount of oxygen consumed during utilization of the first aliquot of DAB gave a DAB to O ratio of 0.43. The second measurement gave 0.48.

Catalase was used in these experiments because of the possibility of peroxysomal contamination of the preparation and the necessity of knowing definitely whether or not catalase activity was present. Catalase would be expected to affect

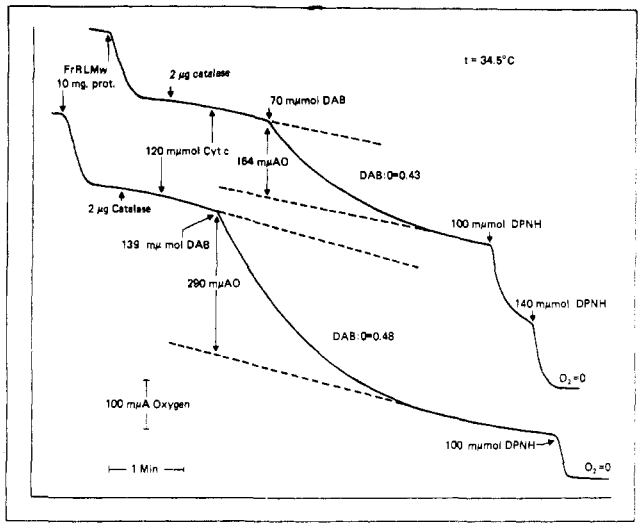



FIGURE 11: Polarographic measurement of stoichiometry between DAB and oxygen. The jacketed 1.7-ml oxygen electrode chamber was used, and the temperature was maintained at 34.5°. Rat liver mitochondria (10 mg of protein) which had been frozen approximately 15 hr and thawed were added to respiration medium, and the reagents were added as shown. The amount of oxygen utilized during oxidation of aliquots of DAB was measured, and oxidation of an aliquot of DPNH served as standard for the oxygen ordinate.

the oxygen stoichiometry of the reaction if hydrogen peroxide was a product or an electron acceptor. Brain mitochondria, which would not be expected to be contaminated with peroxidase (DeDuve and Baudhuin, 1966), also gave a DAB to oxygen ratio between 0.4 and 0.5, in the absence or presence of added catalase. Thus one oxygen molecule ($O_2$) oxidizes one DAB molecule, and the product is not hydrogen peroxide. The four-electron reduction of molecular oxygen to water by DAB agrees with the stoichiometry of approximately four cytochrome *c* molecules reduced per DAB molecule, as reported for Figure 6.

## Discussion

Several lines of biochemical evidence reported here establish that the dye DAB reduces the intramitochondrial cytochrome *c*, thereby supporting respiration. Inhibitor studies with antimycin A and cyanide show that reducing equivalents from DAB enter the respiratory chain on the oxygen side of cytochrome *b* and before cytochrome oxidase. Polylysine inhibition of mitochondrial DAB oxidation provides further confirmation of the involvement of cytochrome *c* and cytochrome oxidase.

Direct evidence for the primary interaction of DAB with cytochrome *c* comes from the *in vitro* demonstation (Figure 6) that purified cytochrome *c* is reduced by DAB in the absence of a mitochondrial preparation. The decrease in mitochondrial DAB oxidation after the mitochondrial have been depleted of cytochrome *c* is added evidence of this interaction.

The Arrhenius plots indicate that at temperatures above 30° the reaction rate ceased to increase with increasing temperature. This phenomenon could result from either a destruction of cytochrome oxidase or from a destruction or loss of cytochrome *c*. At temperatures above 30°, addition of cytochrome *c* to the mitochondrial normalized the increase in DAB oxidation rate as a function of temperature. This observation would indicate that the cytochrome oxidase activity had remained adequate, but that at above 30° the intramitochondrial

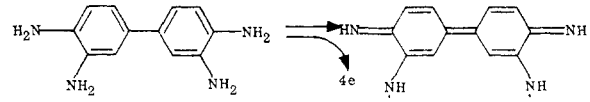



FIGURE 12: Hypothetical reaction for oxidation of DAB to the free radical form.

cytochrome *c* must have been unavailable in a concentration adequate for the expected higher reaction rate. If the cytochrome *c* had leaked out of the mitochondria or had been released from its binding site, its effective concentration at the reaction site would be decreased, resulting in the apparent decrease in activity.

These experiments also indicate that at temperatures above 30° extramitochondrial cytochrome *c* becomes available to the mitochondrial cytochrome oxidase. If this phenomenon has any *in vivo* relevance, it is that reducing equivalents from the extramitochondrial compartment could, by this mechanism, become accessible to the mitochondria. Cytochrome *c* is synthesized extramitochondrially, and, furthermore, the mitochondrial outer membrane (Sottacasa *et al.*, 1967), as well as the endoplasmic reticulum (Strittmatter, 1963), contain cytochrome $b_5$ and NADH cytochrome *c* reductase. At the normal body temperature of 37°, therefore, those reductases may be functional rather than vestigial.

Wojtczak and Sottacasa (1972) showed that isolated outer mitochondrial membranes were impermeable to cytochrome *c*, but their reaction temperatures were not specified. Their discussion postulated free cytochrome *c* between the inner and outer mitochondrial membrane at the salt concentrations present in the intact cell.

The evidence reported here that DAB donates its electrons to the cytochrome *c* of the mitochondrial respiratory chain, combined with the histological evidence (Seligman *et al.*, 1968) that oxidized DAB is deposited on the outer surface of the inner mitochondrial membrane, confirms the picture of the mitochondrial membrane with the reaction site for reduction of cytochrome *c* located on the outer surface of the inner membrane (Racker *et al.*, 1971; Lee, 1970). Recently Reith and Schuler (1972) have also shown that DAB supports mitochondrial respiration *in vitro* and that DAB produces a cyanide-sensitive conformational change in mitochondria.

The reduction of 3.3 or 3.7 cytochrome *c* molecules by DAB agrees with the four-electron oxidation of DAB by molecular oxygen suggested by the DAB:O stoichiometry of 0.5. Thus one can postulate the reaction shown in Figure 12. The radicals would be transient intermediates in the polymerization reaction (Seligman *et al.*, 1968), and one-electron reductions of the radicals found at the ends of the polymer chains would account for the slight deviation from an exact four-electron donation by each molecule of DAB.

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# Regulation of Muscle Contraction. Effect of Calcium on the Affinity of Troponin for Actin and Tropomyosin†

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ABSTRACT: The calcium dependence of the reconstituted actomyosin adenosine triphosphatase (ATPase) depends on the troponin concentration. Reconstituted actomyosin was made from rabbit actin and myosin and increasing amounts of chicken tropomyosin and troponin. The ATPase was measured at different calcium ion concentrations from $1.4 \times 10^{-9}$ to $1.4 \times 10^{-6}$ M in the presence of $Mg^{2+}$ (pH 7.5). When the data are normalized, it is seen that the calcium concentration required for half-maximal activity increases from $3.6 \times 10^{-8}$ to $1.7 \times 10^{-7}$ M with a ninefold increase in troponin concentration. Half-maximal calcium binding by troponin is at $6.5 \times 10^{-8}$ M $Ca^{2+}$ in the presence of 2 mM $MgCl_2$ at pH 7.5. An explanation for the observed shift in calcium dependence is that troponin in the absence of calcium has a higher affinity for actin–tropomyosin than troponin with calcium. This interpretation is supported by a mathematical analysis of this multiple equilibria system in the Appendix accompanying the paper. Measurement of exchange between free and actin-bound troponin is consistent with the assumption that the system is at equilibrium.

Troponin, a regulatory protein in the myofibril, binds calcium and confers a calcium-sensitive inhibition on actomyosin ATPase and superprecipitation. This inhibition is considered the *in vitro* analog of relaxation in the myofibril (Ebashi and Kodama, 1965). Troponin is associated with actin and tropomyosin in the thin filaments of vertebrate striated msucle (Ohtsuki *et al.*, 1967). Calcium stimulates contraction by combining with troponin without any known direct effect on myosin. An important question is how troponin transmits information about the presence or absence of calcium to actin and myosin resulting in contraction (ATPase) or relaxation (inhibition of ATPase), respectively. Kinetic experiments have shown that troponin reduces the affinity of actin for myosin in the presence of EGTA[1] (Parker *et al.*, 1970; Eisenberg and Kielley, 1970) and that troponin's effect is mediated through actin and tropomyosin rather than myosin (Weber and Bremel, 1971; Spudich and Watt, 1971). Structural studies of native and synthetic thin filaments and X-ray diffraction of muscle have shown that in the presence of calcium, tropomyosin lies in the groove of the F-actin helix (Moore *et al.*, 1970; Spudich *et al.*, 1972) and in relaxing conditions it shifts out of the groove possibly to the position of myosin attachment on actin (Hanson *et al.*, 1973; Huxley, 1973; Parry and Squire, 1973).

The present study is concerned with the interaction of troponin with actin, tropomyosin, and myosin with particular attention to the effect of calcium. Evidence is presented which suggests that calcium reduces the affinity of troponin for the actin–tropomyosin complex. It is possible that this change in affinity is a fundamental property of the calcium-sensitive regulation of contraction in the myofibril. The tight binding of troponin to actin and tropomyosin in the absence of calcium may be directly involved in keeping tropomyosin out of the groove of the actin helix, a position found only in the specific conditions of relaxation.

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[1] Abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl-ether)-*N,N'*-tetraacetic acid.