# Antimycin-insensitive Cytochrome-mediated Respiration in Fresh and Aged Potato Slices[1]

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## ABSTRACT

The effect of antimycin A on the respiration of fresh potato (*Solanum tuberosum* var. Russet Burbank) slices has been determined in the presence and absence of *m*-chlorobenzhydroxamic acid (CLAM). Two antimycin-binding sites are indicated. At low concentrations antimycin alone inhibits respiration only slightly. When CLAM and low antimycin are added together, respiration is sharply inhibited, as in response to cyanide. High antimycin alone is as inhibitory as cyanide. The branch point to the alternate path is intact in fresh slices, as is the hydroxamate-sensitive component. The full alternate path is inoperative, however, as indicated by the sensitivity to cyanide. The data suggest an alternate path loop which bypasses the high affinity antimycin site and returns electrons to the cytochrome path. Antimycin at high concentrations prevents articulation of the loop with the cytochrome path.

The respiration of aged slices is not only markedly resistant to antimycin at high concentrations, but quite insensitive to CLAM in the presence of antimycin. A model is proposed which involves parallel paths within complex III of the cytochrome path, with one path bearing the high affinity, and the other the low affinity antimycin site. With slice aging the antimycin affinity of the latter site is even further reduced, providing a relatively antimycin-insensitive bypass to both the high affinity antimycin-sensitive cytochrome path, and the CLAM-sensitive alternate path. The alternate path loop in fresh slices is presumed to feed into the low affinity antimycin-sensitive arm of the cytochrome path.

In the previous paper we reported on the operation of the CN-resistant, or alternate, path in fresh and aged potato (*Solanum tuberosum* var. Russet Burbank) slices (30). Estimation of the contribution of the alternate path under normal conditions depends upon titration of the respiration with an inhibitor of the alternate path, *e.g.* CLAM[3] in the presence and absence successively of an inhibitor of the Cyt path (1). Whereas CN is normally the inhibitor of choice (14, 15) there are occasional experimental reasons to avoid CN, and to this end we sought to use antimycin instead, which is thought to serve within complex III (23, 25), safely beyond the branch point to the alternate path, which is deemed to be in the ubiquinone region (28). We soon observed that antimycin is a poor inhibitor of the Cyt path in aged potato slices, and exerts a dual inhibitory effect on fresh slice respiration at disparate concentrations. Thus, the study of varying antimycin

effectiveness in potato slices, and the meaning thereof, became an end unto itself.

Recently it has been revealed that the binding site of antimycin is not Cyt *b*-562 or *b*-566 as formerly proposed (26, 27) but a distinct nonheme protein of mol wt 11,500 (6) which is one of the subunits of complex III (23). Whereas one antimycin-binding site has been reported for species of yeast and *Neurospora*, and for beef heart and mung bean (8, 10, 14, 25), recent studies by Burger *et al.* with mitochondrial particles from *Schizosaccharomyces pombe* have shown the presence of two antimycin-binding sites. Burger *et al.* (4, 19, 20) have postulated a dual electron transport chain with an antimycin-sensitive site in each branch, one branch containing the *b* cytochromes and the other devoid of Cyt. The branches converge on Cyt *c* and terminate in a common Cyt oxidase.

Two distinct sites for antimycin binding have also been reported by Grimmelikhuijzen *et al.* (9, 10) in a mutant of *Candida utilis*, a CN-sensitive yeast (5). The binding of antimycin to a high affinity site was found to bring about the reduction of the *b* cytochromes without inhibiting $O_2$ uptake, whereas antimycin binding to a low affinity site was observed to inhibit $O_2$ uptake fully. The model proposed was again one of parallel branches of the electron transport chain in complex III, with the *b* cytochromes lying on only one branch.

Finally it is to be noted that much as in aging potato slices, antimycin sensitivity of the Cyt path of the poky mutant of *Neurospora crassa* decreases markedly during development of the alternate path (17). In what follows we examine the characteristics of antimycin effectiveness in fresh and aged potato slices, both with respect to its suitability for the estimation of the contribution of the CN-resistant path to the normal respiration, and with respect to the prospect of a dual pathway containing a bypass of the Cyt *b* region within the Cyt chain.

## MATERIALS AND METHODS

The methods of slice preparation and of measurement of respiratory rates have been described in the preceding paper (30). Stock solutions of CCCP, antimycin A, and HOQNO were made in absolute ethanol. One hundred $\mu$l of the stock solution were diluted with 100 ml of 0.1 mM $CaSO_4$ solution or 0.01 M phosphate buffer (pH 7.3) in 0.1 mM $CaSO_4$ to give the maximum inhibitor concentration used in these experiments. Mitochondria from fresh and aged potato slices were prepared as previously described (21).

**Biochemicals.** CCCP, antimycin A, and HOQNO were from Sigma Chemical Co. CLAM was synthesized as previously noted (30).

## RESULTS

**Effect of Antimycin and HOQNO on Fresh Slices.** Figure 1 shows the titration of respiration in coupled fresh potato slices with antimycin. Antimycin inhibits respiration in two stages with

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[3] Abbreviations: CLAM: *m*-chlorobenzhydroxamic acid; CCCP: carbonyl-cyanide *m*-chlorophenyl hydrazone; HOQNO: 2-heptyl-4-hydroxyquinoline-N-oxide.

increasing concentration—the first to about 30% and the second to more than 70% (cf. 11). Unexpectedly, the antimycin-insensitive respiration observed with low antimycin concentrations is completely abolished by 1 mM CLAM. Whereas the response to low antimycin and CLAM together suggests the presence of the alternate path in fresh potato slices, such is not the case, since the respiration at low antimycin concentrations in the absence of CLAM is inhibited by 0.1 mM CN (Fig. 1).

The results of Figure 1 suggest the presence of two antimycin-binding sites. Binding to the high affinity site diverts electrons to a segment of the alternate path which returns them to the Cyt path beyond the high affinity site. Binding to the low affinity site inhibits electron flux through the loop of the alternate path as well as through the Cyt path, and thereby inhibits respiration to the same degree as CN. HOQNO, an inhibitor of complex III acting at the same site as antimycin (25), inhibits fresh slice respiration in a manner analogous to that of antimycin.

Altogether, the foregoing observations indicate that the branch point and the CLAM-sensitive component are intact in fresh potato slices. There is an impairment of a segment of the alternate path, however, since the respiration remains sensitive to CN.

**Respiratory Rates of Fresh Slices.** The presence of a segment of the alternate path in fresh slices raises the question of its capacity. To determine the capacity of the Cyt path and of the alternate path loop, the respiration was titrated with CLAM in the presence and absence of 0.2 $\mu$M antimycin.

Figure 2 shows that CLAM alone does not affect the respiration. In the presence of 0.2 $\mu$M antimycin titration with CLAM yields a classical inhibition curve indicative of the titration of the alternate path loop. Respiration approaches an asymptote with increasing CLAM in the presence of antimycin which represents the so-called residual respiration, $V_{res}$. Antimycin, 10 $\mu$M, reduces the respiration at once to the residual level and no further effect of CLAM is perceptible. When $V_T$, the total respiration rate, or $V_T'$, the total respiration rate corrected for $V_{res}$, is plotted against g(i), the maximal attainable rate of the alternate loop at a given CLAM

concentration (1, 30), the result is a horizontal line, which indicates that in the absence of antimycin the alternate path loop is not contributing to the respiration. The possibility that CLAM diverts electrons to the Cyt path has been ruled out (30). Table I establishes that $V_T$ is in each case the sum of the Cyt path plus $V_{res}$. That is, in the absence of antimycin there is no contribution by the alternate path loop. The capacity of the alternate path loop can be estimated by CLAM titration in the presence of 0.2 $\mu$M antimycin, and is seen to be about 70% of the uninhibited respiration. On the other hand, 10 $\mu$M antimycin fully suppresses the alternate path loop as well as the Cyt path, and thus inhibits to the same extent as CN (30).

**Effect of Antimycin and HOQNO on Aged Slices.** Figure 3 shows the effect of antimycin on the respiration of aged potato slices in the presence and absence of 1 mM CLAM. The response to HOQNO is the same as to antimycin. The point of major interest is that in the presence of CLAM there is virtually no inhibition by antimycin at low concentrations and very little inhibition at high concentrations. That is to say, aged slice respiration is largely antimycin-insensitive. Nevertheless, at high antimycin concentration in the absence of CLAM antimycin *stimulates* respiration, an indication that antimycin impermeativity is not at issue in antimycin insensitivity, and that a modest Pasteur effect can be elicited in air. When respiration is stimulated above the control rate by high antimycin, CLAM *per se* is mildly inhibitory (Fig. 3), an affirmation that the alternate path is engaged. Uncoupler preempts the stimulatory effect of antimycin at high concentrations with the result that a slight inhibition by high antimycin is observed instead. Taken together the data indicate that antimycin resistance in aged slices is due primarily to the insensitivity of the Cyt path, and secondarily, under special conditions, to a modest diversion of electrons to the alternate path. To rule out the possibility that antimycin insensitivity is due to the impairment of CLAM effectiveness by antimycin, a CLAM titration was done in the presence of antimycin and CN together. It is evident (Fig. 4) that CLAM is fully effective in the presence of antimycin as evidenced by the titration response in the presence of CN. With CN present in the absence of CLAM antimycin has little effect, an affirmation, if such is needed, that the low affinity antimycin-
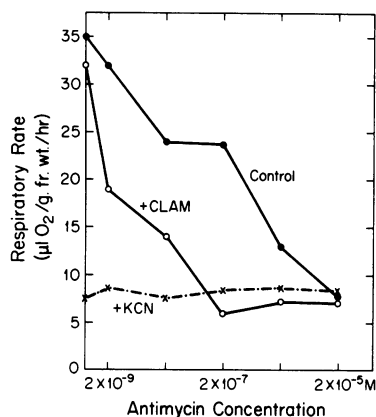


FIG. 1. Effect of antimycin with and without CLAM on respiration of coupled fresh potato slices. CLAM concentration: 1 mM; KCN: 0.1 mM.
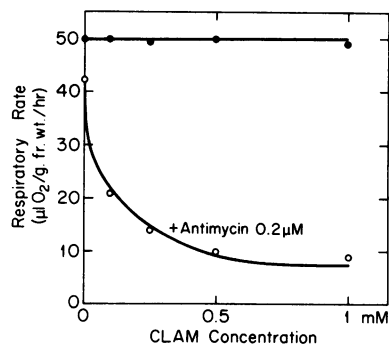


FIG. 2. Effect of CLAM in presence and absence of low antimycin on coupled fresh potato slices.

Table I. Respiratory Components of Coupled Fresh Potato Slices Determined with Low and High Antimycin Concentrations

Experiments 1 and 2 with different tubers. $V'_T = V_{cyt} + \rho\,V_{alt}$, where $\rho$ = fraction of the alternate path which is operating (1). $V'_T = V_T - V_{res}$.

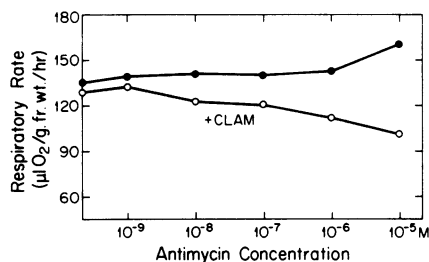| Experiment | Antimycin concentration for determination of g(i) | $V_T$ | $V_{alt(loop)}$ | $\rho$ | $\rho \cdot V_{alt}$ | $V_{cyt}$ | $V_{res}$ |
|---|---|---|---|---|---|---|---|
| No. | $\mu$M | \multicolumn{2}{c}{$\mu$l O$_2$/g fresh wt.hr} | | | \multicolumn{2}{c}{$\mu$l O$_2$/g fresh wt.hr} | |
| 1 | 0.2 | 50 | 34 | 0 | 0 | 41 | 9 |
| 2 | 10 | 37 | 0 | 0 | 0 | 30 | 7 |

FIG. 3. Effect of antimycin with and without CLAM on respiration of coupled aged potato slices. CLAM concentration: 1 mM.
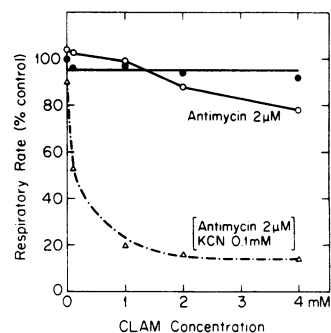


FIG. 4. Synergistic effect of CLAM and antimycin, or antimycin plus CN, on coupled aged potato slices.



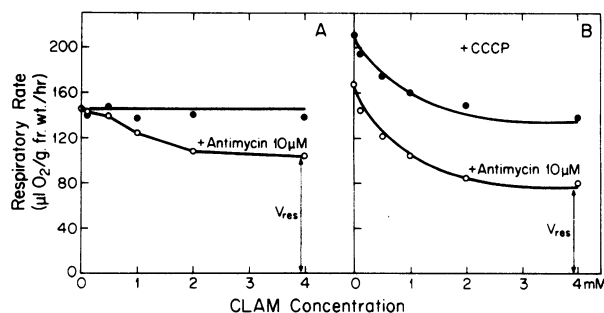FIG. 5. Effect of CLAM in presence and absence of antimycin on respiration of coupled and uncoupled aged potato slices. A: coupled; B: uncoupled.

binding site (Fig. 1) is not on the alternate path. Since 10 $\mu$M antimycin inhibits fresh slice respiration 80%, and exerts very little effect on aged slices even in the presence of CLAM, we are left with the choice of an antimycin bypass (within the Cyt path) and/or a profound drop in antimycin affinity in aged slices.

**Alternate Path Estimation with Antimycin and CLAM in Coupled and Uncoupled Aged Slices.** The estimation of the magnitude of the alternate path by titration with CLAM in the presence of cyanide depends on full inhibition of the Cyt path by CN (1). Inhibition by CLAM is at most 27% in the presence of 10 $\mu$M antimycin (Fig. 5A). Clearly, the alternate path is grossly under-estimated (Fig. 4). In any event, when the erroneously low values of g(i) are plotted against $V_T$ or $V_T'$, the slope of the line is 0. That is, $\rho$, the fraction of the alternate path which is operating—numerically equal to the slope—is 0, and we deduce therefrom that there is no contribution of the alternate path in the absence of inhibitors.

In the presence of uncoupler CLAM is inhibitory both in the absence and presence of antimycin—an indication that the alternate path is contributing to the respiration. When $V_T$ or $V_T'$ is plotted against g(i) in this instance, $\rho = 0.8$, a value which affirms the participation of the alternate path. Graphic analysis shows that the maximum value of g(i), the alternate path, is twice as great in uncoupled as in coupled aged slices.

**Respiratory Path Components in Coupled and Uncoupled Aged Slices.** The elements comprising the respiration of coupled and uncoupled aged slices respectively are shown in Table II. It is evident that the alternate path is engaged only in the presence of uncoupler, and that its apparent value depends upon whether CN or antimycin is used to determine g(i), the maximal value of alternate path activity at given CLAM concentrations in the presence of an inhibitor of the Cyt path.

In coupled slices, $V_{alt}$ estimated with antimycin is about 40% of the value determined with CN—the disparity indicating the ineffectiveness of antimycin in inhibiting the Cyt path. When antimycin is used to inhibit the Cyt path, and the respiration subsequently titrated with CLAM to yield g(i), the graphical representation of $V_T = V_{cyt} + \rho V_{alt} + V_{res}$ plotted following the substraction of $V_{res}$, yields a $V_{cyt}$ that is too low, and a $V_{alt}$ that is too low. $V_{cyt}$ is too low by the extent that $V_{res}$ is too high. That is, $V_{res}$ contains uninhibited $V_{cyt}$ within it. Accordingly, if the true $V_{res}$, as indicated by the respiration which survives the simultaneous presence of CN and CLAM, is subtracted from the apparent $V_{res}$, the antimycin-insensitive component of $V_{cyt}$ is obtained, which, upon addition to the graphically estimated $V_{cyt}$, yields the true $V_{cyt}$ (Table II). When CN is used in the determination of g(i), the graphically obtained values are the true values.

Whereas the alternate path as determined with CN is diminished by uncoupler, the alternate path as determined with antimycin is augmented (Table II). Uncoupler enhances antimycin sensitivity, lessening the bypass around the antimycin-sensitive site in the Cyt path. Accordingly, electron diversion to the alternate path is augmented compared with that in the absence of uncoupler, and $V_{alt}$ approaches the value determined by CLAM titration in the presence of CN. Since in the presence of uncoupler

the absolute value of $V_{alt}$ determined with CN is lower than that determined with antimycin, the low $V_{alt}$ value in the first instance must be due to CN. If g(i) values obtained with CN for uncoupled aged slices are plotted against g(i) values obtained with antimycin, the slope of the line is 0.58, indicating that the alternate path as determined with CN has been decreased 42%. In the presence of uncoupler CN seemingly inhibits the alternate path somewhat, or impairs access to it. In any event, g(i) is underestimated, and for this reason $\rho$ rises above 1. The unduly high observed residual respiration in the presence of uncoupler and antimycin verifies that inhibition by antimycin remains incomplete. Thus, in coupled slices the antimycin-insensitive leg of the Cyt path accommodates 64% of the total Cyt path, while in uncoupled slices 38% of the Cyt path remains antimycin-insensitive.

Finally, the ineffectiveness of antimycin on the respiration of aged slices is verified in isolated mitochondria (Table III). Not only is antimycin less inhibitory than CN, but CLAM fully inhibits the CN-resistant respiration while only partly reducing the antimycin-insensitive respiration.

## DISCUSSION

The titration of fresh slice respiration with antimycin in the presence and absence of CLAM reveals the presence of two antimycin-binding sites. Binding to the high affinity site diverts electrons to a segment of the alternate path which delivers them to the Cyt path beyond the high affinity site. Binding of antimycin to the low affinity site inhibits electron flow via the segment of the alternate path as well as through the Cyt path and thus inhibits respiration to the same extent as CN (30). Moreover, the above observations indicate that the branch point as well as the CLAM-sensitive component are present in fresh potato slices. Hackett et al. (11) first depicted the biphasic nature of antimycin inhibition but did not remark on it.

The respiration of fresh potato slices is in all cases CN-sensitive, which indicates that the hydroxamate-sensitive segment of the

Table II. Respiratory Components of Coupled and Uncoupled Aged Potato Slices

Estimate of values made on the basis of CLAM titrations in the presence of KCN and antimycin respectively. True $V_{res}$ represents respiration in the presence of CLAM and KCN. Antimycin-sensitive respiration estimated graphically. Antimycin-resistant cytochrome path respiration obtained by subtracting true residual from graphically obtained apparent residual respiration.

| g(i) determined with | CCCP 10 μM | $V_T$ | $V_{alt}$ | p | p · $V_{alt}$ | $V_{cyt}$ Antimycin sensitive | $V_{cyt}$ Antimycin insensitive | $V_{cyt}$ Total | $V_{res}^{observed}$ | $V_{res}^{corrected}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | μl $O_2$/g fresh wt.hr | | | | μl $O_2$/g fresh wt.hr | | | | |
| KCN (0.1 mM) | - | 144 | 92 | 0 | 0 | - | - | 105 | 39 | 39 |
| | + | 206 | 51 | 1.1 | 56 | - | - | 106 | 44 | 44 |
| Antimycin (10 μM) | - | 146 | 40 | 0 | 0 | 38 | 69 | 107 | 108 | 39 |
| | + | 211 | 87 | 0.8 | 70 | 60 | 37 | 97 | 81 | 44 |

Table III. Effect of Respiratory Inhibitors on Fresh and Aged Potato Mitochondria

Concentration of addenda as follows: Succinate, 10 mM, ADP, 0.17 mM, ATP, 0.33 mM. Mitochondrial protein was 2.3 mg from fresh tissue, and 2.7 mg from aged slices - in a final volume of 3.4 ml.

| Inhibitor | FRESH | | AGED | |
|---|---|---|---|---|
| | nmol $O_2$/min mg protein | % | nmol $O_2$/min mg protein | % |
| Control | 190 | 100 | 186 | 100 |
| KCN, 0.2 mM | 0 | 0 | 55 | 30 |
| Antimycin A, 1.2 μM | 4 | 2 | 104 | 56 |
| KCN + CLAM, 1 mM | 0 | 0 | 0 | 0 |
| Antimycin + CLAM | 0 | 0 | 55 | 30 |
| Antimycin + CLAM + KCN | 0 | 0 | 0 | 0 |
| Respiratory Control Ratio | 2.77 | - | 3.1 | - |



FIG. 6. Suggested arrangement of electron carriers in mitochondria from fresh and aged potato slices. X: alternate oxidase; P: antimycin-binding protein; $A_1$: high affinity antimycin site; $A_2$: low affinity antimycin site; F: unknown component of complex III. $K_{M,A_2}^{Aged} \gg K_{M,A_2}^{Fresh}$

electron transport chain represents only part of an incomplete alternate pathway, which forms a loop around the high affinity antimycin-binding site. The actual alternate oxidase is either missing or ineffective, and its function is realized only after a period of RNA, protein, and phospholipid synthesis (32, 33). We have taken the view that the spate of lipid breakdown which follows slicing represents the degradation of membrane phospholipids (31). Whatever the reason for the CN sensitivity of fresh potato slices, phospholipid synthesis with aging is a sine qua non for the development of the induced respiration and CN resistance (32, 33) and we tentatively view the reestablishment of CN resistance with aging as due to the intussusception of new phospholipid or lipoprotein components into existing mitochondria (29, 30).

Our interest centers both on the existence of two antimycin-binding sites in fresh potato slices and on the sharp drop in antimycin sensitivity in aged slices. What is the disposition of the two sites? Which site, if not both, changes its affinity for antimycin with slice aging? Several options, examined in turn, are as follows: (a) the high affinity site is on the Cyt path whereas the low affinity site is on the alternate path; (b) the two antimycin sites are in series on the Cyt path; (c) the two antimycin sites are in parallel on the Cyt path.

The first option is reminiscent of the dual respiratory path of the photosynthetic bacterium Rhodopseudomonas capsulata where a Cyt-containing path without Cyt oxidase is 100 times less sensitive to antimycin than the parallel path which ends in Cyt oxidase (18, 34). The main reason for rejecting this option in potato slices is that even at high concentrations antimycin fails to inhibit the respiration of CN-resistant aged slices in the presence of CN. The series model cannot encompass the observation that high concentrations of antimycin stimulate the respiration of aged slices—an indication of inhibition of the Cyt path with an attendant Pasteur effect—while the concomitant addition of CLAM fails to cause marked inhibition (Fig. 3). Thus, we are left with the parallel model which must account both for the duality of antimycin sites and for the diminution of antimycin sensitivity with aging.

The parallel model derives from the suggestions of Grimmelikhuijzen (9) and Burger (4) who have proposed the existence of a
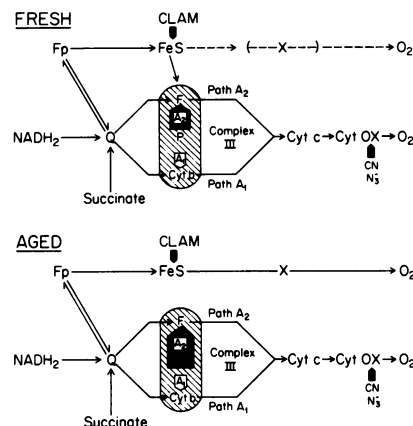
dual electron transport chain in complex III, both arms of which have antimycin-susceptible sites. Figure 6 depicts a tentative scheme of the electron transport chain in fresh and aged potato slices. In fresh slices the alternate path is depicted as incomplete, and an Fe-S center—rather than a Cu or Mo metalloprotein—is takens to be the site of CLAM inhibition, not solely for the reason that hydroxamates complex iron (22, 24), but also because iron (13) and sulfur (12) are absolute requirements for the development of the alternate path, and copper deficiency promotes the development of the alternate path (7). Complex III is viewed as having two branches which converge through Cyt c and terminate in Cyt oxidase. The protein component, P, is the seat of the antimycin-binding sites (9, 23). Recently, Das Gupta and Rieske (6) have shown that a subunit of complex III can be specifically labeled with an antimycin analog, and have accordingly proposed that this subunit bears the antimycin-binding sites.

The binding of antimycin to the high affinity binding site is deemed to inhibit the Cyt b-containing branch (A, in Fig. 6) inasmuch as Grimmelikhuijzen et al. (9) have shown that binding to the high affinity site causes complete reduction of the b cytochromes without affecting $O_2$ uptake. The parallel path through complex III which is susceptible to high antimycin concentrations ($A_2$ in Fig. 6) is deemed kinetically restrictive through its first leg (4). In consequence, in the presence of low concentrations of antimycin, electron traffic is diverted through a loop of the alternate path originating at coenzyme Q (28) and returns to path $A_2$ at a site, F, susceptible to antimycin at high concentrations.

In aged slices the CN-resistant alternate path is functional and the antimycin affinity of site F on the $A_2$ pathway is markedly decreased, since CLAM only partly inhibits the respiration in the presence of high levels of antimycin, whereas CN and CLAM totally inhibit respiration. Since antimycin continues to cause the

reduction of the *b* cytochromes (Arron and Day, unpublished) it seems reasonable that the high affinity antimycin site has not changed its characteristics.

Bonner and Slater (3) have noted the tendency of electrons to leak past the antimycin block in coupled potato mitochondria. That is, when an anaerobic suspension of mitochondria was treated concomitantly with antimycin and a pulse of air, the previously reduced *c* cytochromes as well as Cyt $a$-$a_3$ were first oxidized and then reduced again upon the depletion of $O_2$. The reduction was postulated to occur by way of an antimycin-resistant path. Uncoupler was found to diminish the leak much as uncoupler has been found to diminish the antimycin-insensitive component of the Cyt path in aged potato slices (Table II). The antimycin leak has been demonstrated as well by Estabrook (8) in heart muscle submitochondrial particles where a disparate effect of antimycin on the reduction of the *b* cytochromes and on $O_2$ uptake was noted. Further affirmation of the leak in animal mitochondria is to be had in the observed reduction of Cyt *c* and *a* in the presence of CN and antimycin (8).

The scheme of Figure 6 accounts for the hydroxamate-sensitive loop in the presence of 0.2 $\mu$M antimycin as well as for the diminished sensitivity to antimycin in aged slices. It explains why neither high antimycin alone nor CLAM in the presence of antimycin fails to inhibit respiration of aged slices completely. It explains further why antimycin stimulates aged slice respiration.

Finally, there is the question of whether the cyt *b*-containing branch ($A_1$ path) of the Cyt path in complex III is perhaps selective for electrons from $QH_2$, while the other branch ($A_2$ path) serves to oxidize the semiquinone QH·. Kröger (16) has indicated that the substrate dehydrogenases reduce Q in a stepwise manner, the unstable semiquinone dismutating to yield $Q + QH_2$. Burger *et al.* (4) have shown that the flux via the $A_2$ branch increases in the presence of Lubrol WX, presumably as the result of the stabilization of QH·. Conceivably, the degraded lipoprotein environment in fresh potato slices disfavors operation of the $A_2$ branch, while aging, with the regeneration of an appropriate lipoprotein environment, leads to an increase in QH· stability with an improved likelihood of the $A_2$ branch involvement. In any event, the alternate path is thought to oxidize reduced coenzyme Q (28). A scheme for the apportionment of electrons between the Cyt and alternate paths has been described by Bahr and Bonner (2).

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