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Inhibition by Adenine Derivatives of the Cyanide-Insensitive Electron Transport Pathway of Plant Mitochondria

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ABSTRACT

The effect of benzylaminopurine was studied on cyanide-resistant mitochondria isolated from aged slices of potato tuber (*Solanum tuberosum* L. var. Bintje). Benzylaminopurine specifically acted on the cyanide-resistant alternative pathway. In the case of succinate oxidation, it mimicked the action of salicylhydroxamic acid and restored a good oxidative phosphorylation. Kinetic analyses showed that inhibitions by benzylaminopurine, salicylhydroxamic acid, and disulfiram occurred at mutually exclusive sites on the alternative pathway. Cyanide-resistant malate oxidation was only partially inhibited by benzylaminopurine and this inhibition occurred for low concentrations of this compound. On the other hand, the oxidation of exogenous NADH remained unaffected.

The effects of several adenine derivatives with or without cytokinin activity and that of a purine analog with anticytokinin activity were also studied. The variation in effectiveness to inhibit cyanide-resistant electron transport was: benzylaminopurine and 7-pentylamino-3-methylpyrazolo (4,3 *d*) pyrimidine (anticytokinin) > γ-γ'dimethyl-allyl-adenine > 6-benzoylamino-9-benzylpurine > kinetin > adenine. No correlation was observed between the ability to inhibit the alternative pathway and the biological activity of these compounds. Liposolubility appeared as a major factor for potential inhibitory effect on the alternative pathway.

Aging of potato tuber slices is known to bring about both a rise in tissue respiration and the appearance of cyanide-resistance (11, 15, 28). Mitochondria isolated from aged potato slices are characterized by the presence of a functional cyanide-resistant alternative pathway sensitive to SHAM[1] (5, 6).

The process of aging is often compared to a breaking of dormancy resulting in the enhancement of cellular metabolism (1, 7, 13). As cytokinins are known to stimulate many cellular events, this observation suggests that these compounds might be involved in this process. However, the addition of cytokinins to the aging medium for potato slices prevents the increase in the rates of respiration and phosphate absorption (12, 21). Moreover, several results indicate that cytokinins can be inhibitors of cell respiration (3, 18, 20). Very likely, the site for such an effect is located in the mitochondria (18, 19). The proposed site would allow inhibition of both the alternative pathway and the internal NADH dehydrogenase (19).

This paper describes the effects of BA on the alternative electron transport pathway in mitochondria from aged potato tuber slices. The relations between BA and known inhibitors of the alternative pathway (SHAM, disulfiram) were also investigated. Finally, in order to more precisely define the mode of action of BA, some other compounds, chemically related to BA, were used to establish a possible structure-activity relationship.

[1] Abbreviations: SHAM, salicylhydroxamic acid; BA, benzylaminopurine; DMAA, dimethyl-allyl-adenine; RD 21, 6-benzoylamino-9-benzylpurine; 7 PMP, 7-pentylamino-3-methylpyrazolo (4,3 *d*) pyrimidine; DMSO, dimethylsulfoxide.

MATERIALS AND METHODS

Potato tubers (*Solanum tuberosum* L., var. Bintje) were purchased locally. Slices, 1 mm thick and 1 cm in diameter, were cut out of the central part of the tubers and aged during 24 h as previously described (6). Mitochondria from fresh and aged slices were isolated and purified according to conventional procedures (6). Mitochondrial proteins were measured by acid digestion and nesslerization.

O_2 uptake was measured at 25°C in a 2-ml Plexiglas cell fitted with a Clark electrode. The medium was 300 mM mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer (pH 7.2), and 1 mg/ml BSA. Depending on the substrate used (10 mM succinate, 30 mM malate, 1 mM NADH), 0.5 to 1 mg of mitochondrial protein was used.

KCN in water solution neutralized to pH 7.2 and SHAM (Aldrich) dissolved in ethanol or DMSO were used as inhibitors of the Cyt and the cyanide-resistant alternative pathway, respectively. The inhibition of the alternative pathway was also studied with disulfiram (Sigma) dissolved in ethanol. BA was from Fluka, kinetin and adenine were from Calbiochem, DMAA was from Sigma. RD 21 was a generous gift from Dr. Dat-Xuong and 7 PMP was kindly supplied by Dr. Laloue. All adenine derivatives and the purine analog 7 PMP were dissolved in DMSO.

The lipophilicity of adenine and 7 PMP was experimentally determined at pH 7 from partition coefficients in octanol:water (16). Apparent partition coefficients for other adenine derivatives were calculated according to the method of the additivity of the hydrophobic fragmental constants (22).

RESULTS

Effect of BA on Succinate Oxidation. Succinate oxidation by mitochondria isolated from fresh potato slices showed a good phosphorylative capacity (Fig. 1A). The addition of BA during state 3 rate had no effect on the O_2 uptake and the oxidation was totally inhibited by cyanide. By contrast, mitochondria isolated from aged potato slices also actively oxidized succinate but the oxidation was loosely coupled to phosphorylation, since respiratory control and ADP:O values were rather low (Fig. 1B). The addition of 500 μM KCN only partially inhibited O_2 uptake (49%) because of the presence of a cyanide-resistant electron transfer. A subsequent addition of BA at a concentration of 750 μM almost totally inhibited the residual succinate oxidation. When BA was added first, during state 3, succinate oxidation was only slightly inhibited (17%) (Fig. 1C), and the subsequent addition of KCN stopped all O_2 uptake.

Thus, BA seems to inhibit the cyanide-resistant part of succinate

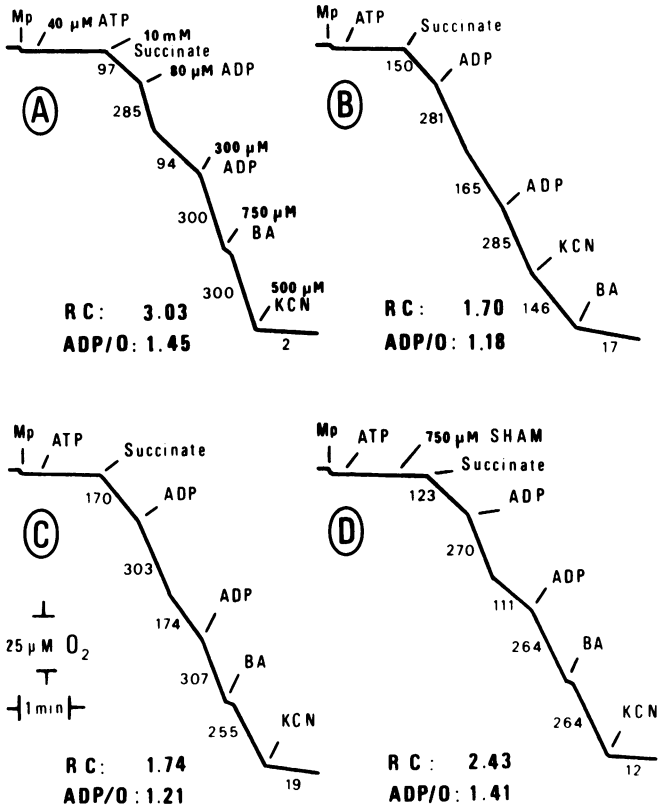

FIG. 1. Effect of BA on succinate oxidation by mitochondria isolated from fresh (A) and aged (B, C, D) potato slices. Numbers along the traces are O_2 uptake in nmol/min·mg protein.

oxidation mediated through the alternative pathway that is present in aged potato mitochondria, whereas it does not inhibit the cyanide-sensitive Cyt pathway in fresh potato mitochondria. However, to rule out the possibility of any inhibitory effect of this compound on the Cyt chain in aged potato mitochondria, SHAM, a well-known inhibitor of the alternative pathway (25), was added before succinate (Fig. 1D). Under these conditions, succinate oxidation was mediated only through the Cyt pathway, as proved by the higher respiratory control and ADP:O values obtained. The addition of BA during state 3 did not inhibit the O_2 uptake, and a further addition of KCN totally blocked succinate oxidation. It must be added that the inhibition of the alternative pathway by a given level of BA was found to be independent of the mitochondrial protein concentration and was easily reversible by washing BA-treated mitochondria with buffer.

Table I summarizes the effects of BA and SHAM on the oxidative and phosphorylative capacities of aged potato mitochondria. As previously observed, BA and SHAM inhibited the total state 3 rate of succinate oxidation to the same extent. The cyanide-resistant electron transfer (V_{alt}) was slightly less inhibited by BA than by SHAM. But the respiratory control and ADP:O ratios, which were rather low in the absence of inhibitors, reached normal values when either BA or SHAM was present. All these results show that BA specifically acts on the cyanide-resistant alternative pathway during succinate oxidation, in the same way as SHAM does.

Influence of the Nature of the Substrate. A characteristic feature of the cyanide-resistant, SHAM-sensitive pathway in aged potato mitochondria is that it is used by electrons from succinate and malate but not by those from exogenous NADH (5, 6).

Table II indicates the effects of BA and common electron transport inhibitors on the oxidation of the three substrates. KCN partially inhibited succinate and malate oxidations, whereas ex-

Table I. *Effect of BA and SHAM on the Oxidative and Phosphorylative Properties of Mitochondria from Aged Potato Slices*

Condition	V_T[a]	V_{alt}[b]	RC	ADP:O
	nmol/min·mg protein		*ratio*	
Control	305	160	1.75	1.17
BA, 750 μM	259	22	2.25	1.35
SHAM, 750 μM	268	11	2.33	1.42

[a] Total rate of succinate oxidation in state 3.

[b] Rate through the alternative pathway, measured in the presence of 500 μM KCN.

Table II. *Effect of KCN, SHAM, and BA on Succinate, Malate and NADH Oxidations by Mitochondria From Aged Potato Slices*

The sensitivity to the inhibitors was expressed as percentage of inhibition of the state 3 rate before the addition of the inhibitor. The mean values, for state 3 rates, obtained from four independent experiments and expressed in nmol O_2/min·mg protein, were 270 for succinate, 67 for malate, and 274 for exogenous NADH.

Inhibitor	Inhibition		
	Succinate	Malate	NADH
	%		
KCN, 400 μM	54	35	94
SHAM, 750 μM	14	20	2
BA, 750 μM	17	17	0
KCN + SHAM	98	92	95
KCN + BA	93	60	95
KCN + BA + SHAM	99	94	96

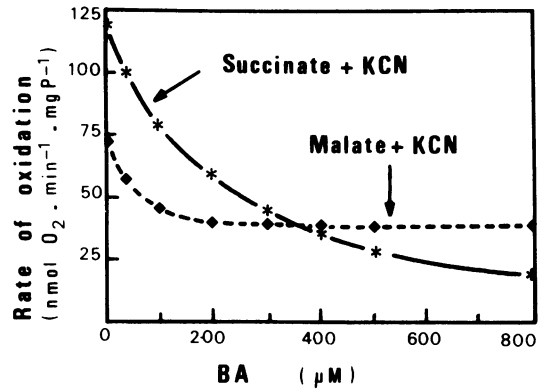

FIG. 2. Effect of BA on the cyanide-resistant succinate and malate oxidations in mitochondria from aged potato slices. The Cyt pathway was inhibited by 500 μM KCN.

ogenous NADH oxidation was almost totally cyanide-sensitive. SHAM and BA inhibited the total electron transfer to the same extent (15–20%) with succinate or malate as substrates, but had no effect on the oxidation of exogenous NADH. In the presence of KCN, an addition of SHAM nearly blocked the electron transfer with all substrates. BA mimicked the effect of SHAM in the case of succinate oxidation, but with malate, the dual addition of KCN and BA only brought about a 60% inhibition. A further addition of SHAM was necessary to achieve complete inhibition.

A more detailed study of the action of BA on the cyanide-resistant fraction of succinate and malate oxidations is shown in Figure 2. The cyanide-resistant part of succinate oxidation can be gradually inhibited to the extent of 80% by raising the BA concentration to 800 μM. By contrast, with the cyanide-resistant part of malate oxidation, there was an immediate but limited inhibition in the presence of low concentrations (200 μM) of BA. Higher

concentrations of BA did not increase the inhibition, which remained stable at a level of 48%. As a result, in the presence of cyanide and BA, malate oxidation still allows a substantial rate of cyanide-resistant electron transport whereas succinate oxidation nearly disappears.

Comparison of BA and SHAM Effects on Succinate Oxidation. Since the rate of succinate oxidation is affected in a similar way by SHAM and BA (Table II), the question arises as to the identity of their sites of action. A kinetic study of the inhibition of succinate oxidation via the alternative pathway (in the presence of KCN) is shown in Figure 3. The results, drawn as Dixon plots, show that the value of the apparent inhibition constant (K_i) was higher with BA (200 μM) than with SHAM (110 μM). This difference between the two constants was always observed even when the absolute values of K_i varied slightly (*cf.* Fig. 4B).

An attempt was made to obtain more precision regarding the sites of action of these two inhibitors. The interaction between the two compounds was followed on cyanide-resistant succinate oxidation by varying BA concentrations in the presence of increasing concentrations of SHAM (Fig. 4A). The curves obtained clearly show that the high cyanide-resistant electron transfer was almost totally inhibited by 1 mM BA, the presence of increasing concentrations of SHAM strengthening the inhibitory effect of BA substantially.

Using a Dixon plot, a series of lines were obtained which were convergent on the horizontal axis (Fig. 4B). The obtaining of nonparallel lines indicates that SHAM and BA have independent binding sites (4). Furthermore, the linear Dixon plot observed in the absence of SHAM show that inhibition of the alternative pathway by BA occurs at a single site.

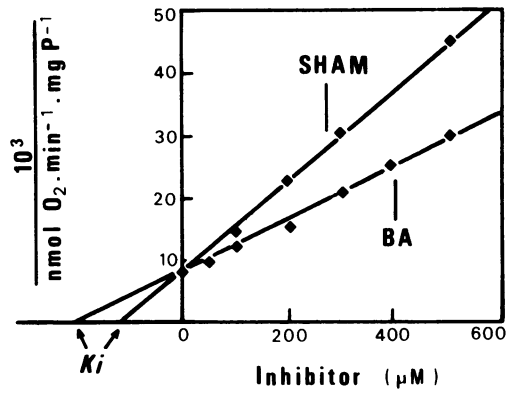

FIG. 3. DIXON plot of BA and SHAM inhibitions of the cyanide-resistant electron transfer in mitochondria from aged potato slices. Mitochondria oxidized succinate in the presence of 500 μM KCN.

It was shown recently that disulfiram, a potent inhibitor of the alternative pathway, does not interact with SHAM in the inhibition process (10). Inasmuch as a similar observation is made with BA, the relation between BA and disulfiram was investigated in the case of cyanide-resistant succinate oxidation. A Dixon plot with varying disulfiram concentrations in the absence or presence of increasing concentrations of BA is shown in Figure 5. Two series of conclusions can be drawn from this Figure. First, the low K_i value obtained with disulfiram (7.5 μM) contrasted with the high values observed with SHAM or BA (Fig. 3). Second, the nonparallel lines proved that disulfiram and BA do not have the same site of action when inhibiting the alternative pathway.

Effect of Various Adenine Derivatives. To assess the generality of the inhibitory properties of BA towards the alternative pathway, the effects of several compounds, structurally related to BA, were studied. Among them there were compounds with cytokinin activity (kinetin, DMAA), without cytokinin activity (RD 21, adenine) or even with anticytokinin activity (7 PMP). Identical concentrations (750 μM) of the different compounds were used, which all appeared to produce a maximal effect in this case. The different levels of inhibition obtained with the various adenine derivatives were highly variable. As previously observed, BA inhibited the alternative pathway to 85% (Fig. 2). DMAA and RD 21 inhibited to 62% and 42%, respectively, whereas kinetin inhibited to 32%. Adenine was the least effective, showing only a 9% inhibition. All the compounds were thus less active than BA itself, with the exception of the cytokinin antagonist 7 PMP whose effect was of the same magnitude (90%) as that of BA.

As it appears that substances having a strong cytokinin activity (BA) or an anticytokinin activity (7 PMP) can act in a similar manner, a study of their interaction on the alternative pathway was investigated. The linear Dixon plot of the cyanide-resistant succinate oxidation *versus* 7 PMP concentration in the absence of BA (Fig. 6) showed the existence of a single site of action for 7 PMP and gave a K_i of 120 μM, close to that observed with SHAM (Fig. 3). The family of lines obtained with fixed levels of BA were roughly parallel with that obtained in the absence of BA (Fig. 6). This indicates that 7 PMP and BA act on mutually exclusive binding sites.

Finally, in terms of inhibitory activity, the various compounds can be arranged in the following order: BA, 7 PMP > DMAA > RD 21 > kinetin > adenine. Since this classification seems to be rather far from the known hormonal activities of the compounds, liposolubility was envisioned as a better explanation. The apparent partition coefficient of adenine in octanol:water was found to be very low (0.11). The coefficients for all other adenine derivatives, calculated from the value of the adenine nucleus, according to Rekker (22), showed that kinetin was the least liposoluble (1.71).

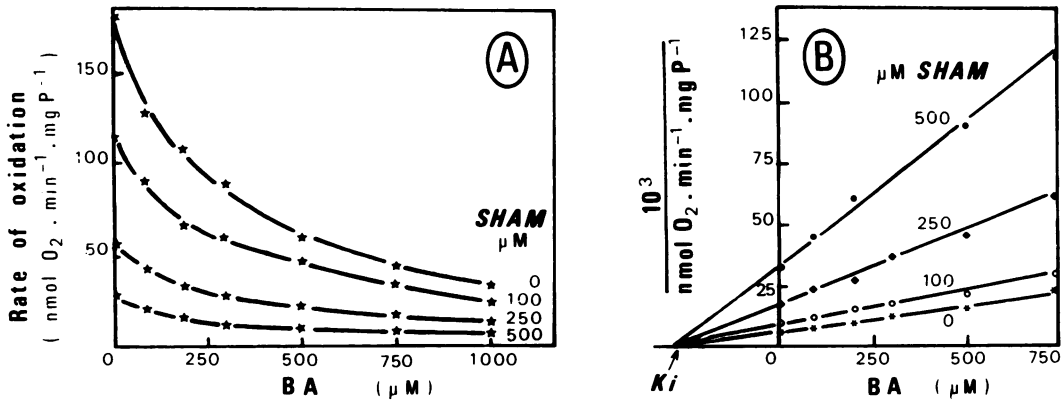

FIG. 4. A, interaction of BA and SHAM on the cyanide-resistant electron transfer in mitochondria from aged potato slices. Mitochondria oxidized succinate in the presence of 500 μM KCN. BA was added in the presence of increasing concentrations of SHAM. B, DIXON plot of BA binding in the presence of SHAM. Experimental values are from A.

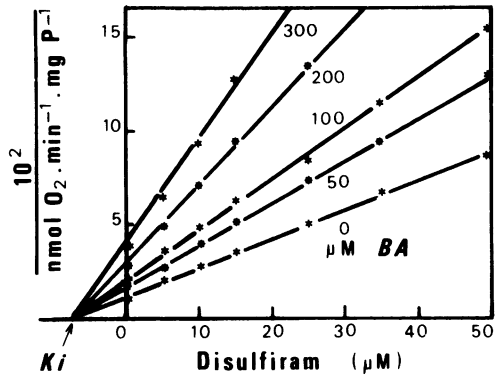

FIG. 5. DIXON plot of the inhibition of the cyanide-resistant electron transfer by disulfiram in the presence of increasing concentrations of BA. Mitochondria from aged potato slices oxidized succinate in the presence of 500 μM KCN.

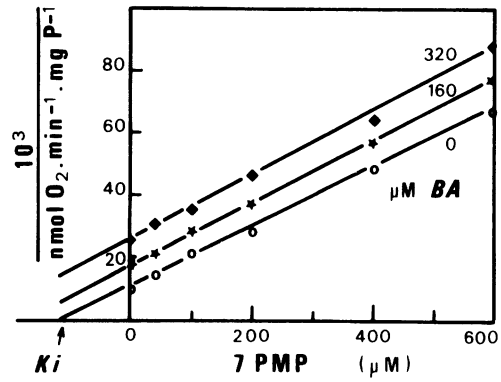

FIG. 6. DIXON plot of the inhibition of the cyanide-resistant electron transfer by 7 PMP in the presence of increasing concentrations of BA. Mitochondria from aged potato slices oxidized succinate in the presence of 500 μM KCN.

The partition coefficients of BA and DMAA were similar (2.10) whereas that of RD 21 was the highest (3.54). As for the purine analog 7 PMP, it appeared very liposoluble since the experimentally found coefficient was 3.15. As a matter of fact, the distribution of the compounds according to their lipophilicity is roughly parallel to their inhibitory action on the alternative pathway. The slight discrepancy found for RD 21 is probably due to the steric occupation of this compound for which the two substituents on the adenine nucleus could reduce its permeation in the membranes.

DISCUSSION

During succinate oxidation by aged potato mitochondria, the action of BA on electron transport mimics that of SHAM. Thus, BA inhibits specifically electron transport by the cyanide-resistant alternative pathway. A similar action of SHAM and BA on succinate oxidation has been observed previously with mitochondria isolated from callus tissue (18) and stems and hypocotyls of various seedlings (19). As expected, the oxidation of exogenous NADH, which is not mediated by the alternative pathway in aged potato mitochondria (5), is not sensitive to BA.

On the other hand, malate oxidation is only slightly inhibited by BA in comparison with the effect found in mitochondria from seedlings (19). In the presence of cyanide, the electrons from malate use the alternative pathway which is inhibited to 48% by BA. This occurs at low concentrations of the compound and increasing the concentration of BA does not raise the extent of inhibition. By contrast, SHAM totally blocks the cyanide-resistant fraction of malate oxidation (Table II). This result indicates a marked difference between the effects of BA and SHAM on the cyanide-resistant electron transfer from malate to O_2. Such a result is not observed with mitochondria from seedlings where BA and SHAM inhibit the alternative pathway (in the presence of antimycin) to the same extent (19).

This discrepancy in BA effect on malate oxidation observed between different plant mitochondria could originate from the possible various routes offered to the electrons. Malate oxidation can take place either through malate dehydrogenase linked to a rotenone-sensitive NADH dehydrogenase and the Cyt chain, or through malic enzyme linked to a specific NADH dehydrogenase and the alternative pathway (24). Specific conditions (pH, cofactors), naturally occurring or experimentally devised, could allow the preferential use of one pathway to the prejudice of the other. This could explain the discrepancy mentioned above and at least makes the scheme of Miller imperfect (19). This scheme includes only one site for BA inhibition and does not integrate the most recent results on the respiratory chain in plant mitochondria (2, 7, 15, 24).

The detailed study of the interaction of BA and SHAM in the case of succinate oxidation shows that both compounds act on independent binding sites in the alternative pathway. On the other hand, the results presented here show that BA and disulfiram, another inhibitor of the alternative pathway, have also independent binding sites. Moreover, disulfiram does not act at the same site as SHAM (10). These results suggest that there are at least three independent sites of inhibition in the alternative electron transport. As a consequence, the inhibitors of the alternative pathway can be classified presently into three different groups: SHAM, disulfiram, and BA. Propyl gallate, which inhibits the alternative pathway at the same site as SHAM (26), can be attached to the first group.

The study of the action of various adenine derivatives and a purine analog (7 PMP) reveals a gradient in the degree of inhibition of the cyanide-resistant succinate oxidation: BA, 7 PMP > DMAA > RD 21 > kinetin > adenine. The order of effectiveness seems to be roughly parallel to the biological activity detected by various bio-assays in the case of BA, DMAA, kinetin and adenine (17, 23, 27). However, the results obtained with other compounds show that the relationship between the effect on electron transport and the regulation of plant growth is rather ambiguous. RD 21, which is ineffective in most biological assays (23), inhibits the alternative pathway more strongly than kinetin. Moreover, 7 PMP, an anticytokinin (9), is the most powerful inhibitor of the cyanide-resistant electron transfer. Finally, it should be mentioned that zeatin, a naturally occurring cytokinin, has almost no effect on the alternative pathway (18, 19).

These observations suggest a particular action of adenine derivatives on mitochondrial activity, which is not related to the cytokinin effect. The differences in cytokinin activity between the compounds have been related to unequal affinities for a hypothetical receptor site depending on the structure of the adenine derivative (23). For example, acylation and substitution at the 9-position on the basic purine nucleus (RD 21) decreases or suppresses biological activity. On the contrary, this modification only slightly weakens the inhibitory effect on the alternative pathway. In consequence, it may be suggested that the order of effectiveness of the various compounds on electron transport could be rather related, at least for a part, to their degree of liposolubility. In fact, it is easy to establish a link between the low degree of liposolubility of zeatin and adenine and their lack of effectiveness on the cyanide-resistant pathway. Kinetin, which is more active on the electron transfer, is also more liposoluble. The degree of liposolubility of the other adenine derivatives are rather similar although their inhibitory efficiencies on cyanide-resistant pathway are somewhat different. Furthermore, the purine analog 7 PMP, which is the most active on the alternative pathway, is also the more

liposoluble.

Finally, the fact that the inhibition of the alternative pathway can be roughly related to the liposolubility of these compounds suggests a binding site rather inaccessible in the mitochondrial membrane. At this point, it was recently shown that a high cytokinin-binding affinity was present in a mitochondrial fraction isolated from *Phaseolus aureus* (14). This binding activity of the cytokinins occurred only in the presence of a low level of the nonionic detergent Triton X-100, suggesting that a binding moiety is present on the inner surface of the mitochondrial membrane. Moreover, Triton X-100, used at a very low concentration, is also known to produce an inhibition of the alternative pathway in mitochondria from aged potato slices, without altering membrane integrity (8).

A compilation of all these results suggests that adenine derivatives with high liposolubility could enter the mitochondrial membrane and interact with a hypothetical receptor in a lipophilic area, bringing about an interruption in the cyanide-resistant electron transport pathway.

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