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FUSCIN, AN INHIBITOR OF MITOCHONDRIAL SH-DEPENDENT TRANSPORT-LINKED FUNCTIONS

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SUMMARY

1. Fuscin, a mould metabolite, is a colored quinonoid compound which reacts readily with –SH groups to give colorless addition derivatives.

2. Binding of fuscin to mitochondria has been monitored spectrophotometrically. Fuscin binding is prevented by –SH reagents such as *N*-ethylmaleimide, *N*-methylmaleimide, mersalyl or *p*-chloromercuribenzoate. Conversely, fuscin prevents the binding of –SH reagents as shown with *N*-[^{14}C]ethylmaleimide. Once bound to mitochondria, fuscin is not removable by washing of mitochondria.

3. High affinity-fuscin binding sites (K_d = 1 μM, n = 4–8 nmoles/mg protein) are present in whole mitochondria obtained from rat heart, rat liver, pigeon heart or yeast (*Candida utilis*). They are lost upon sonication but are still present in digitonin inner membrane + matrix vesicles. On the other hand, lysis of mitochondria by Triton X-100 does not increase the number of high affinity binding sites indicating that all these sites are accessible to fuscin in whole mitochondria. The number of fuscin high affinity sites appears to correlate with the glutathione content of mitochondrial preparations.

4. Fuscin as well as *N*-ethylmaleimide and avenaciolide are penetrant SH-reagents; they react with intramitochondrial glutathione.

5. Fuscin interferes with the ADP-stimulated respiration of mitochondria on NAD-linked substrates, several functions of the mitochondrial respiratory apparatus being inhibited by fuscin in a non-competitive manner, but to various extents: (a) The electron transfer chain (K_i in the range of 0.1 mM); (b) the lipoamide dehydrogenase system (K_i = 5–10 μM); (c) the transport systems of phosphate ($K_i \approx$ 20 μM) and of glutamate (K_i = 3–5 μM); (d) the ADP transport, indirectly ($K_i \approx$ 10 μM).

6. Like *N*-ethylmaleimide, fuscin inhibits the glutamate–OH^- carrier, the inhibition of that carrier bringing about an apparent increase of aspartate entry in glutamate-loaded mitochondria by the glutamate–aspartate carrier.

7. The inhibition of phosphate transport by fuscin probably accounts for the inhibition of the reduction of endogenous NAD by succinate in intact pigeon heart mitochondria.

Abbreviations: DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid), PCMB, *p*-chloromercuribenzoate

8. By binding the –SH groups of mitochondrial membrane specifically unmasked by addition of micromolar amounts of ADP, fuscin, like *N*-ethylmaleimide, prevents the functioning of ADP translocation.

9. Because of their specific and analogous effects on some well defined mitochondrial functions such as glutamate transport and ADP transport, fuscin and *N*-ethylmaleimide can be distinguished from other –SH reagents. The lipophilic nature of fuscin and *N*-ethylmaleimide which accounts for the accessbility of these compounds to hydrophobic sites in the mitochondrial membrane or on the matrix side of this membrane may be partly responsible for their characteristic inhibitory effects on mitochondrial functions.

INTRODUCTION

Fuscin, a quinonoid compound[1] (Fig. 1) with antibacterial activity, isolated from the culture filtrates of *Oidiodendron fuscum* Robak[2,3] has been reported[4,5] to inhibit the respiration of mitochondria. Besides an effect on the respiratory chain, fuscin interferes with the transport of some anions (phosphate, dicarboxylates, glutamate) in mammalian or yeast mitochondria, the glutamate entry being the most sensitive to fuscin as shown in preliminary reports[6–8].

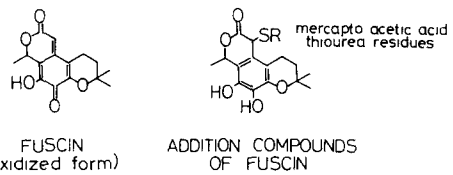

Fig 1 Structure of fuscin established by Barton and Hendrickson[1].

Fuscin which is a colored compound characterized by a strong peak of absorption in the 360-nm region gives colorless addition derivatives with –SH groups[1,2,7] This reaction is easily monitored by a decrease in absorbance of fuscin. The decoloration of fuscin has been used to estimate the amount of fuscin which binds to mitochondria or mitochondrial fragments[7] as compared to other –SH reagents

This paper gives an account of the observed effects of fuscin on some mitochondrial functions (electron transport, ATPase activity, anion transport). It shows that fuscin competes with other –SH reagents such as mersalyl, *N*-ethylmaleimide or *p*-chloromercuribenzoate (PCMB) for binding to mitochondria. The correlation between binding to –SH groups and inhibitory effects is discussed together with the interrelationships between the different mitochondrial functions which are inhibited

MATERIALS

Fuscin extracted from *Oidiodendron fuscum* Robak was a generous gift of Professor D. H. R. Barton (Imperial College of Science and Technology, London). It was used in solution either in ethanol or in dimethylformamide. When added to a

0.25 M sucrose solution the peak maximum was at 355 nm at pH 6 5, 365 nm at pH 7.4 and 368 nm at pH 8. The binding tests of fuscin to mitochondria were generally carried out in isotonic sucrose solution (0.25 M) buffered at pH 7.4 with Tris–HCl; fuscin binding was assessed by the disappearance of the peak in the 360-nm region, as measured in a dual wavelength Aminco–Chance spectrophotometer using the pair 374–385 nm, to avoid interference with NAD(P)H spectrum.

L-Glutamate, ATP, ADP, lipoamide and mersalyl were purchased from Sigma (St Louis, Mo , U.S.A.); NADH, yeast glutathione reductase (EC 1.6.4.2), lipoic acid, lipoamide dehydrogenase (EC 1.6.4.3) and glutathione, from Boehringer (Mannheim, Germany), *N*-ethylmaleimide and digitonin (A grade) from Calbiochem (Los Angeles, Calif., U.S.A.); *N*-[^{14}C]ethylmaleimide and [^{32}P]phosphate from C.E.A. (Saclay, France); rotenone from Aldrich (Milwaukee, Wisc , U.S.A.); 2,6-dichloroindophenol from Fluka (Switzerland).

Radioactivity was measured either in a gas flow counter (RA-15 Intertechnique, France) or in a scintillation counter (SL-30 Intertechnique, France).

METHODS

Rat liver mitochondria were isolated in 0.27 M sucrose buffered with 1 mM Tris–HCl, at pH 7.4 (ref. 9). Sonicated fragments were prepared at 0 °C by submitting mitochondria in 0 27 M sucrose to sonic oscillation in a Branson sonifier at maximum output for periods of 30 s. Unbroken mitochondria were removed by centrifugation at 25 000 $\times g$ for 15 min. Sonicated fragments were recovered from the supernatant by centrifugation at 100 000 $\times g$ for 30 min. Inner membrane+matrix vesicles were obtained either according to Schnaitman and Greenawalt[10] by digitonin treatment or according to Parsons and Williams[11] by treatment with 20 mM phosphate. pigeon heart and rat heart mitochondria were prepared in 0.225 M mannitol–0.075 M sucrose, 0.02 M Tris–HCl (pH 7.6). 0.2 mM EDTA according to Chance and Hagihara[12], and tested in the same buffer with EDTA omitted (MST medium), and yeast mitochondria (*Candida utilis*) according to Balcavageand Mattoon[13].

Reduced and oxidized glutathione were estimated by the method of Tietze[14] based on the catalytic action of GSH or GSSG in the reduction of Ellman reagent (5,5′-dithio-bis-(2-nitrobenzoic acid DTNB), by a mixture of NADPH and yeast glutathione reductase. The adduct of *N*-[^{14}C]ethylmaleimide and glutathione present in a trichloroacetic acid extract of mitochondria was isolated by thin-layer chromatography[15] The same type of solvents can allow the separation of fuscin–glutathione adduct stainable by ninhydrin.

Dihydrolipoic acid and dihydrolipoamide were obtained by reduction by borohydride[16]: Lipoic acid or lipoamide is dissolved in methanol, water is added in the ratio 1 : 1 and then solid borohydride. After a few minutes at room temperature, the pH is brought to 1–2 by addition of HCl to destroy the excess of borohydride and the solution neutralized to pH 6 with HCO_3^- or NaOH.

The rate of reduction of endogenous NAD(P) was measured in a dual-wavelength spectrophotometer at 20 °C. The two wavelengths chosen (355 nm *minus* 380 nm) were in a symmetrical position with regard to the maximum of the absorption peak of free fuscin in order to avoid optical interference of the fuscin spectrum and the NAD(P)H spectrum

RESULTS

Reactivity of fuscin towards –SH compounds Binding of fuscin to mitochondria

In the earlier studies, fuscin had been shown to bind to –SH compounds. Michael[2] obtained stable cristalline addition complexes with mercaptoacetic acid in which fuscin and mercaptoacetic acid were in equimolar amounts Birkinshaw *et al.*[3] obtained with thiourea a compound, fuscin thiouronium chloride, which in contact with water decomposed rapidly into its constituents Barton and Hendrickson[1], who established the quinonoid structure of fuscin, gave the structure of these derivatives of fuscin. We have extended these studies to other –SH compounds of biological interest. The amount of fuscin which reacts with cysteine, mercaptoethanol, glutathione, dihydrolipoamide, dihydrolipoic acid and dithiothreitol is most conveniently assessed by measuring the disappearance of the peak in the 360-nm region (see Methods). When using the above mentioned –SH compounds in equimolar amounts with respect to fuscin, the half time of decoloration was 30 s–1 min at 20 C, but on standing in an open cuvette, the 360-nm peak reappeared after a lag phase and at a rate which varied according to the –SH compound tested. In the case of cysteine, the lag period was less than 1 min and the half time of recoloration was of the order of 3 min. The decoloration of fuscin by dihydrolipoamide was stable for at least 30 min allowing an easy determination of the stoichiometry of the reaction; 1 mole of dihydrolipoamide was consumed per mole of fuscin added. The stability of fuscin derivatives with glutathione, dithiothreitol and mercaptoethanol was intermediate, the lag period for partial recoloration of fuscin being of the order of 5–10 min Based on these observations, it was assumed that decoloration of fuscin which occurs on addition of mitochondria was due to reaction of fuscin with SH compounds present in mitochondria. To test this hypothesis we carried out a titration of free mitochondrial –SH groups by fuscin in the absence and in the presence of SH reagents such as *N*-ethylmaleimide, mersalyl, *N*-methylmaleimide and PCMB To avoid interference due to turbidity the differential absorbance change in the reaction of fuscin with mitochondria was measured with a dual-wavelength spectrophotometer using the wavelength pair 375–385 nm (see Methods). Scatchard plots of binding data for fuscin and for *N*-[^{14}C]ethylmaleimide are presented in Fig. 2. Fuscin binding was prevented by *N*-ethylmaleimide (Fig. 2A) and conversely *N*-[^{14}C]ethylmaleimide binding was prevented by fuscin (Fig. 2B). Other typical SH reagents such as PCMB, mersalyl, and *N*-methylmaleimide behaved like *N*-ethylmaleimide. These data afford evidence that fuscin bind to mitochondrial –SH groups. Avenaciolide, an inhibitor of glutamate transport[17] used in the accompanying paper[18] comparatively with fuscin was also found to prevent fuscin binding to mitochondria.

The curvature of the Scatchard plot for fuscin binding (Fig 2A) may be indicative of several different types of sites. Taking the minimal hypothesis of two non interacting sites, the curve in Fig. 2A may be decomposed into two straight lines corresponding to high affinity fuscin binding sites ($K_d < 1\ \mu M$, $n = 6$ nmoles per mg protein) and low affinity sites ($K_d > 30\ \mu M$). The number of high affinity sites varied according to mitochondrial preparations and ranged from 4–8 nmoles per mg protein for rat liver, rat heart and pigeon heart mitochondria A linear relationship was found between the amount of fuscin bound and the amount of protein used (Fig. 3) The high affinity sites were still present in digitonin inner membrane

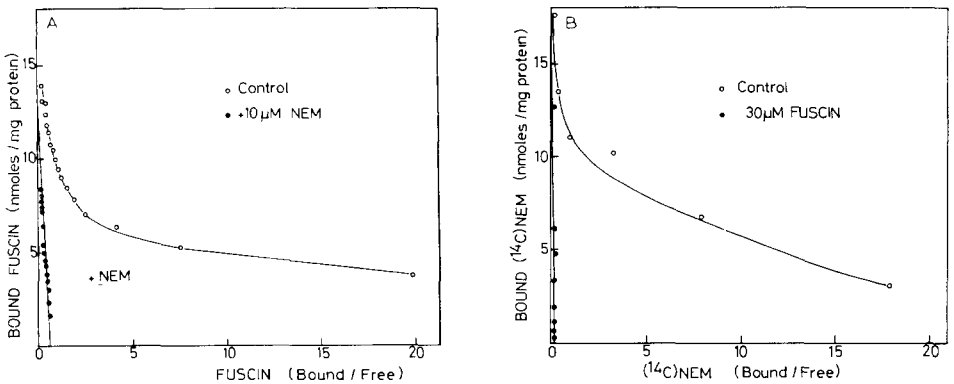

Fig 2 Competition between fuscin and *N*-ethylmaleimide for binding to mitochondria (A) Rat heart mitochondria (2 mg protein) were suspended in 3 ml of MSTE medium (*cf.* Methods) pH 7 4, containing 5 μM rotenone, 2 μg/ml oligomycin and 0.8 μg/ml antimycin at 20 °C Successive addition of fuscin in solution in *N*, *N'*-dimethylformamide were made at 4 min intervals The binding of fuscin was assessed by measuring the disappearance of the colour of free fuscin at 375 nm *minus* 385 nm in a dual-wavelength spectrophotometer (Chance–Aminco) in the absence or the presence of 10 μM *N*-ethylmaleimide (NEM) The straight line obtained in presence of *N*-ethylmaleimide was also found with other –SH reagents such as mersalyl, PCMB, *N*-methylmaleimide and also with avenaciolide. (B) Rat liver mitochondria (3 7 mg protein) in 0.25 M sucrose–2 mM Tris–chloride, pH 7 4 and increasing concentrations of *N*-[^{14}C]ethylmaleimide ranging from 1.2 μM–500 μM were incubated for 5 min at 20 C in a final volume of 5 ml When fuscin was used, mitochondria were preincubated for 2 min with fuscin prior to the addition of *N*-[^{14}C]ethylmaleimide [[^{14}C]NEM]

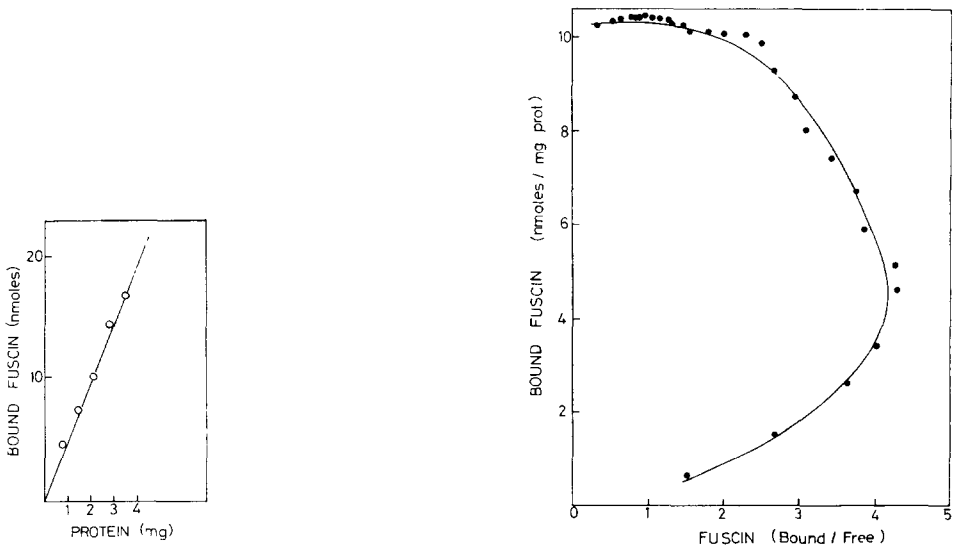

Fig 3 Titration of mitochondria by fuscin. Same conditions as in Fig 2A Fuscin concentration 33 μM.

Fig. 4 Binding of fuscin to yeast mitochondria (*C. utilis*). 2 mg protein of yeast mitochondria were suspended in 3 ml of 0 6 M mannitol–1 mM Tris–HCl, pH 7 4 Addition of fuscin from 0 7 μM to 30 μM Same conditions of assay as in Fig 2

matrix particles prepared from rat liver mitochondria[10] but were lost in sonicated fragments of liver or heart mitochondria (from rat or from pigeon)[7] as well as in the inner membrane particles prepared according to Parsons and Williams[11]. With yeast mitochondria the fuscin binding showed co-operative interactions and a saturation plateau corresponding to about 10 nmoles of fuscin bound per mg protein (Fig. 4). Rat liver microsomes displayed fewer high affinity sites for fuscin than mitochondria (about 2 nmoles/mg protein). The high reactivity of fuscin towards mitochondria is noteworthy in view of its low reactivity towards common proteins such as serum albumin. For instance, bovine serum albumin exhibits only low affinity fuscin-binding sites ($K_d = 40\ \mu M$, $n = 35$ nmoles per mg protein). Lysis of mitochondria with Triton X-100 did not increase the total number of high affinity fuscin-binding sites, indicating that, in intact mitochondria, they are accessible to fuscin. It must be stressed that fuscin-treated mitochondria are not modified in their morphology as indicated by electron microscope controls.

A possible candidate for binding fuscin with high affinity in mitochondria is reduced glutathione. Glutathione which readily reacts with fuscin represents the main non-protein thiol in mitochondria[19,20]. Furthermore, some correlation was found in titration experiments between the fuscin high affinity sites and glutathione in mitochondria or in microsomes (Table I). For instance, the number of high affinity sites in rat liver mitochondria roughly estimated from Scatchard plots (Fig. 2)[7] is of the same order as the amount of reduced glutathione (4–8 nmoles per mg protein). In isolated inner membrane vesicles prepared according to Parsons and Williams[11] which have lost their high affinity binding sites for fuscin the content of reduced glutathione dropped to 0.2 nmole per mg protein. On the other hand, digitonin fragments retain both high affinity fuscin-binding sites and reduced glutathione. In microsomes, the amount of reduced glutathione and high affinity binding sites for fuscin was 3–4 times less than in mitochondria.

Proof that fuscin reacts with mitochondrial glutathione was obtained in the following experiments. Rat liver mitochondria are preincubated with SH reagents

TABLE I

GLUTATHIONE CONTENT OF SUB-CELLULAR FRACTIONS AND FUSCIN HIGH AFFINITY BINDING SITES

N D , not detected Values are given in nmoles/mg protein

*Tissue preparation**	*GSH*	*GSSG*	*High affinity fuscin binding sites***
Rat liver			
Mitochondria	6 4	0 4	5
Digitonin fragments	3.4	0 2	4
Sonicated fragments	0 3	0 1	N D
Inner memb. + matrix vesicles	0 2	N D	N D
Microsomes	2 0	0 6	0 8
Yeast (*S. cerevisiae*)			
Mitochondria	4 0	1 5	–

* *cf.* Methods

** Determined from a Scatchard plot as shown in Fig 2

TABLE II

FREE GLUTATHIONE CONTENT OF RAT LIVER MITOCHONDRIA TREATED WITH –SH REAGENTS

Rat liver mitochondria (28 mg protein) were incubated in 15 ml of 0.12 M KCl, 0.1 mM EDTA, 40 mM Tris-chloride, pH 7.4, in the presence of 20 μM of the –SH reagents (except ferricyanide which was 2 mM), then centrifuged The pellet was washed with 5 ml of 0 27 M sucrose–2 mM Tris–HCl, pH 7.4 and extracted with trichloroacetic acid. The glutathione content was determined in the trichloroacetic acid extracts by the method of Tietze[14] N.D., not detected.

Expt. No.	*Incubation conditions*	*Additions*	*GSH*		*GSSG*
			nmoles/mg protein	*Per cent*	*nmoles/mg protein*
1	10 min at 20 °C	Nil	4 7	100	0 4
		Avenaciolide	1 3	28	0 1
		Fuscin	1.3	28	0 3
		N-Ethylmaleimide	0 3	6	0 2
2	10 min at 20 °C	Nil	5 1	100	0 3
		Mersalyl	4.9	96	0.2
		Ferricyanide	5 1	100	0 3
		2,6-Dichloro-indophenol	N D.	0	2 2
3	30 min at 0 °C	Nil	4.4	100	0.1
		N-Ethylmaleimide	0 3	7	0 2
		Mersalyl	4 0	91	0 1

in isotonic conditions, centrifuged and their content in reduced and oxidized glutathione analyzed in a trichloroacetic acid extract[14] (Table II). 20 μM fuscin or *N*-ethylmaleimide strikingly decreased the content of free reduced glutathione while mersalyl, PCMB or DTNB had only a slight effect related perhaps to the amount of damaged mitochondria (if we assume that they did not react because they could not reach the matrix space). Incubation with fuscin markedly diminished the amount of GSH but did not significantly modify the amount of oxidized glutathione. This effect was compared to that of two oxidizing agents, ferricyanide and 2,6-dichloroindophenol. 2,6-dichloroindophenol which was most often used as oxidant is also able to conjugate with glutathione[21,22] and with mitochondrial membrane thiol groups[23]. As shown in Table II, 2,6-dichloroindophenol penetrates the matrix space and oxidizes part of glutathione while ferricyanide does not lower the amount of intramitochondrial GSH. This is consistent with the fact that 2,6-dichloroindophenol being uncharged at neutral pH is probably readily penetrant whereas ferricyanide does not enter mitochondria[24].

The binding of *N*-ethylmaleimide to intramitochondrial reduced glutathione upon incubation of mitochondria with *N*-ethylmaleimide was confirmed by using *N*-[^{14}C]ethylmaleimide and isolating by chromatography the adduct product *N*-[^{14}C]-ethylmaleimide–glutathione[15]. The same observation holds for fuscin. Using the same chromatographic system as for *N*-ethylmaleimide–glutathione separation, it was possible to isolate a compound which is likely the conjugate of fuscin and glutathione.

Effect of fuscin on NADH oxidase activity

As previously reported, fuscin inhibits the electron transfer in the first site region of the respiratory chain between NADH and the branching of the succinic dehydrogenase[4]. This is in line with the implication of several types of –SH groups in the functioning in this region of the respiratory chain[25]. In the present study, aimed merely at defining the conditions in which fuscin, as an –SH reagent, can be used to dissect mitochondrial function, we were essentially interested in comparing the NADH respiratory chain to other SH-dependent mitochondrial systems for their reactivity and accessibility towards fuscin.

The effect of fuscin was tested on submitochondrial particles obtained by sonication or digitonin treatment to avoid the permeability barrier to NADH Using digitonin inner membrane *plus* matrix vesicles, fuscin, at 10 nmoles per mg protein (final concentration 25 μM) inhibited the NADH oxidation by 30–50 %. Mersalyl at the same concentration brought about the same effect. When sonicated particles from pigeon heart were used, a higher concentration of fuscin (90 μM or 495 nmoles per mg protein) was required to bring about 50 % inhibition of the O_2 consumption (Table III). These observations correlate with the binding studies reported above which indicate that high affinity binding sites for fuscin were still present in digitonin inner membrane + matrix vesicles while they were lost upon sonication. With sonicated submitochondrial particles from yeast (*Saccharomyces cerevisiae*), 170 nmoles fuscin per mg protein (50 μM) had to be used to bring about 50 % inhibition of NADH oxidation

TABLE III

FUSCIN INHIBITION OF NADH OXIDATION BY SONICATED PIGEON HEART MITOCHONDRIA

Sonicated pigeon heart mitochondria were preincubated at 25 C for 3 min in 3 3 ml of MST medium containing 4 mM phosphate with fuscin at different concentrations as indicated in the table Then 0 6 mM NADH (final concentration) was added. The oxygen uptake was measured with a GME oxygraph equipped with a Clark electrode

Fuscin		*Rate of respiration*	
μM	*nmoles/mg protein*	O_2 μM/*min*	%
0	0	60	100
30	166	55	92
60	333	45	75
90	495	30	50
120	633	15	25

In summary, the sensitivity to fuscin of NADH oxidation depends upon the type of mitochondrial particles used and seems to be linked to the glutathione content of those particles. In digitonin mitochondrial particles which still contain glutathione and have high affinity sites for fuscin (Table I) the NADH oxidation is inhibited by low concentration of fuscin, it is the contrary for sonicated fragments.

Inhibition by fuscin of the mitochondrial oxidation of NAD-linked substrates

It was already known that in mammalian mitochondria fuscin at concentrations

as low as 30 μM totally inhibits the oxidation of NAD-linked substrates such as β-hydroxybutyrate[26], pyruvate *plus* malate, glutamate but not that of succinate or ascorbate[4]. Since the respiratory chain NADH . . . O_2 *per se* is not severely affected by fuscin at these concentrations (Table III), it was hypothesized that more reactive sites towards fuscin could be located either at the level of some substrate transport systems in mitochondrial membranes or at the level of specific dehydrogenases.

TABLE IV

FUSCIN INHIBITION OF ENDOGENOUS NAD(P) REDUCTION BY EXOGENOUS SUBSTRATES

The conditions were those of Fig 5 except that malate was replaced by 0.3 mM malonate when the substrate was oxoglutarate and was omitted when the substrate was glutamate. The K_i values were determined by the method of Dixon from the initial rates of endogenous NAD(P) reduction.

Substrate added	*Mitochondria*	K_i (μM)	*Type of inhibition*
Pyruvate (+malate)	rat liver	5	non competitive
	rat heart	8	non competitive
Oxoglutarate (+malonate + phosphate)	rat heart	12	non competitive
Glutamate	rat liver	3	non competitive
	pigeon heart	5	non competitive

A possible interaction of fuscin with mitochondrial NADH-linked dehydrogenases has been assessed by measuring the rate of reduction of endogenous NAD(P) by different exogenous substrates in the presence or absence of fuscin. Fuscin inhibited in a non-competitive manner the reduction of NAD(P) by pyruvate, oxoglutarate and glutamate; the K_i values ranged between 3 and 12 μM (Table IV). In a comparative study, it was found that mersalyl was markedly less effective than fuscin in inhibiting the reduction of NAD(P) by pyruvate (Fig. 5).

Although direct proof is not easy to obtain, it is possible that fuscin reacts with some essential –SH components of the dehydrogenase system of α-ketoacids; a likely candidate is the system lipoamide–lipoamide dehydrogenase. This was suggested by assays carried out with mitochondria lysed by Triton X-100 or with purified preparations of lipoamide dehydrogenase (Fig. 6) showing an increased rate of the reduction of lipoic acid (or lipoamide) in the presence of fuscin, as if fuscin was able to trap the reduced lipoic acid formed. Mersalyl had no effect and this correlates with the absence of inhibition by mersalyl, of pyruvate dehydrogenation (Fig. 5) The sensitivity of the reduction of endogenous NAD(P) by glutamate (Table IV) is not explained by a direct effect of fuscin on glutamate dehydrogenation or transamination. In fact, fuscin at 50 μM does not modify the activity of free glutamate dehydrogenase either when the mitochondrial glutamate dehydrogenase has been made free by lysis of mitochondria with Triton X-100 or by sonication or when a crystalline preparation was used. Neither did fuscin affect the aminoaspartate transferase tested after lysis of mitochondria with Triton X-100.

In line with experiments dealing with the inhibition by fuscin of the mitochondrial respiration, the effect of fuscin on the reduction of endogenous NAD(P)

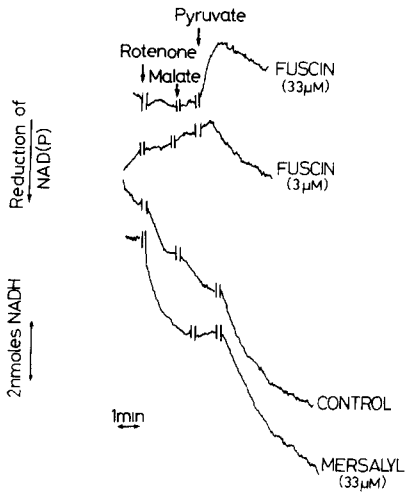

Fig. 5. Inhibition by fuscin of the reduction of endogenous NAD(P) by pyruvate Rat heart mitochondria (2 8 mg protein) were preincubated for 5 min at 20 °C in 3 ml of 0.12 M KCl, 20 mM Tris–HCl, 5 mM phosphate, 5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and fuscin or mersalyl at the indicated concentration Final pH 7 2 Then 10 μM rotenone was added and after 2 min 33 μM L-malate and 8.3 mM pyruvate The rate of reduction of NAD(P) was measured in a dual-wavelength spectrophotometer at 20 °C.

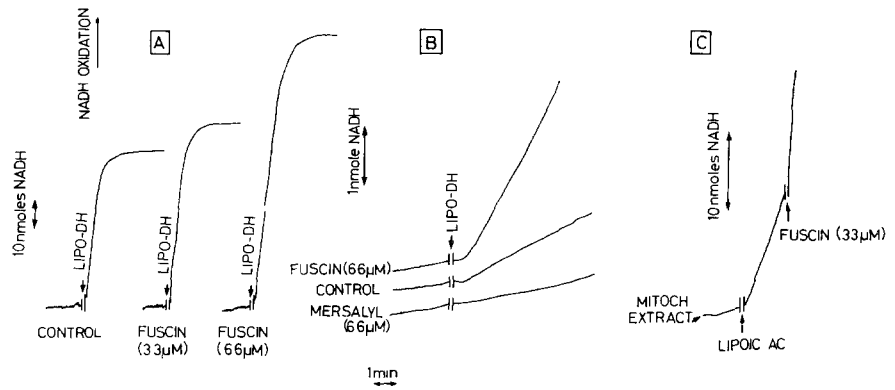

Fig 6 Effect of fuscin and mersalyl on the rate and extent of NADH oxidation by lipoic acid Traces A and B The incubation medium contained in 3 ml, 0.08 M potassium phosphate, pH 5 9 1 mM EDTA, 2 mM NADH, 0 1 mM NAD and as indicated fuscin or mersalyl. Temperature 20 °C. Oxidation of NADH was initiated by addition of 10 μl of a diluted solution of lipoamide dehydrogenase (LIPO-DH) and followed at 340 nm Trace C Same medium as in traces A and B Purified lipoamide dehydrogenase was replaced by a mitochondrial extract (0.5 mg protein) obtained after sonication of rat heart mitochondria in MST medium and high speed centrifugation

by succinate was investigated with pigeon heart mitochondria. The reduction of endogenous NAD upon addition of succinate[27] was found to be, in some preparations of pigeon heart mitochondria, strictly dependent upon the addition of phosphate and not affected by oligomycin The rate of NAD reduction was markedly decreased when the concentration of added phosphate was lower. The half-maximum effect of phosphate was given by a concentration of the order of 300 μM which is in the range of the K_M for the entry of phosphate in mitochondria[28]. The effect of fuscin on the reduction of endogenous NAD(P) was tested in the presence of 330 μM phosphate Fuscin delayed at 1 μM (2.3 nmoles/mg protein) or totally prevented at 4 μM (9 2 nmoles/mg protein) the reduction of NAD(P) by succinate. The inhibitory effect of fuscin on the reduction of NAD by succinate in a phosphate-supplemented medium can be tentatively explained in terms of an indirect action on phosphate transport (see below). It is known that the functioning of the respiratory chain of mitochondria is coupled to an efflux of protons into the surrounding medium[29]. As discussed by Papa *et al* [30], the electron flow along the respiratory chain is linked to phosphate uptake through the flux of protons in the mitochondrial membrane and in turn phosphate uptake imposes an energy load on the redox pressure of the respiratory chain. Based on the above reasoning and on the fact that the oligomycin-insensitive reduction of NAD by succinate depends on the electron flow from succinate to O_2, it can be understood that a partial inhibition of phosphate uptake, as it occurs with fuscin (see below), can markedly affect the reduction of NAD by succinate.

Inhibition by fuscin of anion transport

Several methods described originally by Chappel[31] are currently used to assess the penetration of mitochondrial membrane by anions. They are based on: (1) the osmotic behaviour of mitochondria and their swelling in isotonic ammonium salts of permeant anions, (2) the change in the redox state of intramitochondrial nicotinamide nucleotides depending on the accessibility of substrate anions to their intramitochondrial specific dehydrogenases, (3) the uptake of radioactively labelled anion.

(*a*) *Glutamate transport.* The glutamate transporter has been studied by Chappell and his co-workers[31,32], by Meijer *et al.*[33] and in this laboratory (accompanying paper[18]). We report here some data pertaining to the inhibitory properties of fuscin as compared to other inhibitors such as avenaciolide, a specific inhibitor of glutamate transport[17], *N*-ethylmaleimide[33] or mersalyl.

By measuring photometrically the swelling of rat liver mitochondria in 0.1 M ammonium L-glutamate, fuscin was shown to inhibit glutamate entry[18], the efficiency of the inhibitors being, in decreasing order. avenaciolide, fuscin, *N*-ethylmaleimide, mersalyl while the reverse order is found for the inhibition of swelling of mitochondria in 0.1 M ammonium phosphate[18]. The measure of the initial rate of reduction of endogenous NAD(P) by L-glutamate in a double beam spectrophotometer as described in ref. 7 and in legend of Fig. 7 gave, for rat liver mitochondria, the following results: $K_i^{\text{fuscin}} = 3\ \mu$M, $K_i^{N\text{-ethylmaleimide}} = 18\ \mu$M, $K_i^{\text{mersalyl}} = 70\ \mu$M, the inhibition being non competitive with the three reagents.

Azzi *et al.*[32] suggested the simultaneous presence of a glutamate–hydroxyl carrier and of a glutamate–aspartate carrier, exchanging 1 mole of glutamate against 1 mole of aspartate. The blocking of the first carrier should favour the second one,

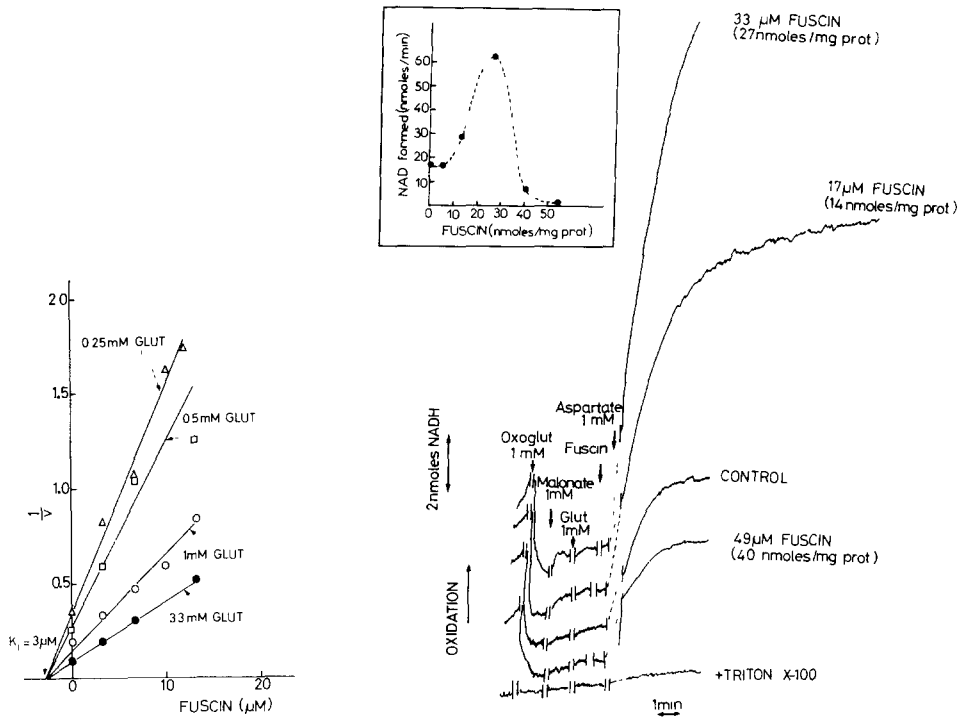

Fig 7 Inhibition by fuscin of the reduction of endogenous NAD(P) by glutamate. Graphical determination of inhibitor constant by the method of Dixon. The reciprocals of initial rates of NAD(P) reduction (expressed in nmoles NAD(P)H formed per min) upon addition of increasing amounts of glutamate are plotted against fuscin concentration Rat liver mitochondria (2 mg protein) were preincubated for 3 min at 20 °C in 3 ml of 0 27 M sucrose-2 mM Tris–chloride, pH 7 45, 3 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone with or without fuscin then 5 μM rotenone was added and, after 30 s the reduction of pyridine nucleotides was started by addition of glutamate

Fig. 8 The activating effect of fuscin on the oxidation of intramitochondrial NAD(P)H by aspartate in the presence of glutamate. As described by Azzi *et al* [32] rat liver mitochondria (3.7 mg protein) were suspended in 3 ml of a medium containing 1 mM phosphate, 80 mM KCl and 20 mM Tris–chloride, pH 7.4 After 2 min, 2 μg antimycin A was added and then the substrates and fuscin as indicated on the figure The reoxidation of endogenous NAD(P)H was measured in a dual-wavelength spectrophotometer using the pair 355–380 nm to avoid interference with fuscin absorption. Temperature 20 C.

that is the exchange between aspartate and glutamate. Using the method of Azzi *et al.*[32] based on the measure of the oxidation of intra-mitochondrial NAD(P)H by aspartate after loading the mitochondria with glutamate, we observed (Fig. 8), in the presence of fuscin, a stimulation of the intramitochondrial NAD(P)H oxidation with a maximal effect at about 25 nmoles fuscin/mg protein. This can be interpreted as a stimulation of aspartate entry through the aspartate–glutamate carrier resulting from the blocking of the glutamate–OH^- carrier by fuscin. Mersalyl which is a very potent inhibitor of phosphate transport, but hardly inhibits glutamate transport *per se*, did not exhibit the effect shown by fuscin.

(*b*) *Phosphate transport.* When assayed by conventional techniques (swelling

in isotonic ammonium phosphate[31], direct uptake of [^{32}P]phosphate, efflux of phosphate generated by hydrolysis of ATP inside mitochondria[34], phosphate transport was found to be blocked by fuscin although less efficiently than by mersalyl. A preincubation of the inhibitor with mitochondria lower significantly the amount of inhibitor required to bring about 50 % inhibition of the phosphate transport. This explains the apparent difference in sensitivity of phosphate transport to fuscin in the experiments on [^{32}]Phosphate uptake reported thereafter where mitochondria have been submitted to a preincubation (Fig. 9) and in the swelling experiments reported in the accompanying paper[18] where no preincubation was carried out.

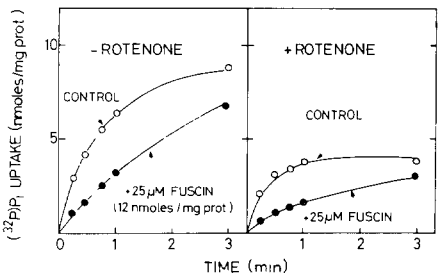

Fig 9 Effect of fuscin on the uptake of [^{32}P]phosphate in rat liver mitochondria Rat liver mitochondria (4 mg protein) were preincubated for 2 min at 20 °C then for 4 min at 0 °C with 2 ml of 0 12 M KCl, 0 01 M Tris–chloride, 0.1 mM EDTA, 10 μg oligomycin and, where indicated 5 μM rotenone and fuscin Final pH 7.2. The incubation at 0 °C was started by addition of [^{32}P]-phosphate in 0 1 ml to a final concentration of 500 μM and stopped by addition of 5 mM mersalyl immediately followed by rapid centrifugation at 0 °C. Control incubations were carried out where mersalyl was added immediately before [^{32}P]phosphate The amount of [^{32}P]phosphate incorporated in the mitochondrial matrix was calculated after deduction of [^{32}P]phosphate found in control incubations.

Assays on [^{32}P]phosphate uptake were carried out at 0 °C in the presence or absence of rotenone as a respiratory inhibitor (Fig. 9). The maximum amount of phosphate incorporated under equilibrium was markedly higher in the absence of rotenone (approx. 10 nmoles phosphate/mg protein) than in its presence (4 nmoles phosphate/mg protein). In spite of this difference, the rates of [^{32}P]phosphate uptake calculated from the first order rate constant values were only slightly higher without rotenone (10 nmoles · min^{-1} · mg protein^{-1}) than with rotenone (8 nmoles · min^{-1} · mg protein^{-1}) with a K_m of about 0.3 mM. In both cases, fuscin was equally inhibitory and at 25 μM (12 nmoles fuscin/mg protein) it inhibited the initial rate of phosphate uptake up to 60–70 %. Under the same conditions, 25 μM *N*-ethylmaleimide produced not more than 5 % inhibition, half inhibition being given by 100 μM *N*-ethylmaleimide. Fuscin inhibition was not competitive with respect to phosphate The uptake of phosphate anions, charge-compensated by an uptake of protons, is stimulated by the mitochondrial respiration and *vice-versa*[30]. The inhibition by rotenone of phosphate accumulation in mitochondria is apparently indirect and results from the specific effect of rotenone on the respiratory chain. In contrast, fuscin which was equally active in the presence or in the absence of rotenone, apparently blocks the transport of phosphate by interacting with the phosphate carrier rather than with the respiratory chain.

(*c*) *ADP transport.* The mitochondrial ADP transport is not sensitive *per se* to small concentrations of –SH reagents. However, when mitochondria are preincubated with *N*-ethylmaleimide *plus* a minute amount of ADP, they cannot catalyse further the exchange of external ADP with internal adenine nucleotides[35,36]. The same observation holds for fuscin but not for other SH reagents such as mersalyl or PCMB As shown in Table V a preincubation of rat liver mitochondria with as little as 10 μM fuscin *plus* 10 μM ADP markedly inhibited (more than 60 %) the rate of ADP translocation whereas fuscin or ADP added separately in the preincubation medium have virtually no effect. When ADP in the preincubation medium was replaced by other nucleotides which are not transported in mitochondria (UDP, CDP, GDP), the ADP transport was not inhibited. The effect of ADP in the preincubation medium was shared by ATP or by adenine analogues which are transported in mitochondria adenosine methylene diphosphate or adenosine hypophosphate). These data point to the nucleotide specificity and indicate that the effect of preincubated ADP (or ATP) is brought about through their interactions with the adenine nucleotide carrier. As postulated in previous papers reporting a similar inhibition of the ADP transport subsequent to preincubation with *N*-ethylmaleimide and ADP[35,36], binding of external ADP to its specific carrier induces the unmasking of a thiol group which is trapped by the SH-reagent. This SH group may belong to a mobile ADP carrier or to membrane components close to the ADP carrier and undergoing a change of conformation upon the binding of ADP. Whereas fuscin inhibits the ADP transport system indirectly, it does not alter the coupling mechanism for ATP synthesis, nor the ATPase activity of sonicated rat liver mitochondria at 200 μM.

Complementary experiments were carried out with [^{35}S]atractyloside a specific inhibitor of the ADP translocation which binds to the ADP carrier or in its

TABLE V

SYNERGIC EFFECT OF FUSCIN AND ADP ON THE ADP TRANSLOCATION

Rat liver mitochondria (4 mg protein) were preincubated in 5 ml of 110 mM KCl, 0 1 mM EDTA and 10 mM Tris–sulfate, pH 7 2 for 2 min at 20 °C with ADP, UDP, CDP, GDP and fuscin as indicated After cooling at 2 °C, [^{14}C]ADP in 200 μl was added to a final concentration of 200 μM The incubation lasted for 30 s at 2 °C and was stopped by addition of 4 μM carboxyatractyloside followed by rapid centrifugation. The amount of [^{14}C]ADP incorporated in the matrix space was calculated from the amount of [^{14}C]ADP present in the pellet after correction for the [^{14}C]ADP in the sucrose space.

No	*Additions in preincubation medium*	[^{14}C]*ADP translocation (nmoles/min per mg protein)*
1	Nil	6.3
2	10 μM ADP	6 2
3	10 μM Fuscin	5 6
4	40 μM Fuscin	3 5
5	10 μM ADP+10 μM Fuscin	2 2
6	10 μM ADP+40 μM Fuscin	1 3
7	10 μM UDP, CDP, GDP	6 0–6 6
8	10 μM UDP+10 μM Fuscin	5.2
9	10 μM CDP+10 μM Fuscin	5 4
10	10 μM GDP+10 μM Fuscin	5 6

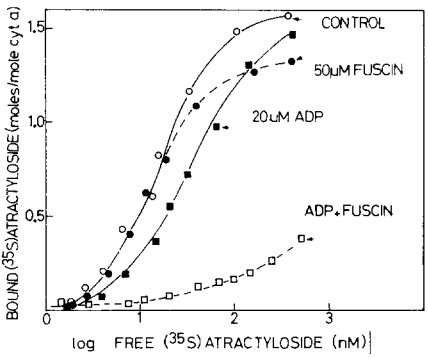

Fig 10 Decrease of [^{35}S]atractyloside binding affinity to rat liver mitochondria upon simultaneous addition of fuscin and ADP. Rat liver mitochondria (9 mg protein) were preincubated in 10 ml of 110 mM KCl, 10 mM Tris–sulfate, pH 7.4, 0.1 mM EDTA, with either 50 μM fuscin or 20 μM ADP, or both or none of them for 2 min at 20 °C. After cooling at 2 °C, [^{35}S]atractyloside was added at concentrations ranging from zero to 0.5 μM. The incubation lasted for 45 min at 2 °C and was ended by centrifugation. The pellets were dissolved in formamide at 180 °C and the radioactivity counted by scintillation. cyt a, cytochrome *a*.

close neighbourhood in the inner membrane[37,38]. As shown in Fig. 10, preincubation of mitochondria with a small concentration of ADP or of fuscin did not alter the [^{35}S]atractyloside binding curve. However, when ADP and fuscin were present together, they prevented [^{35}S]atractyloside binding very efficiently. These data corroborate those obtained on the ADP transport (Table V) and may be interpreted in the same manner.

(*d*) *Transport of the Krebs cycle anions.* Fuscin does not modify the rate of entry of malate[7] nor that of isocitrate when assayed by the reduction of intramitochondrial pyridine nucleotides[26]. Complementary experiments carried out on the transport of [^{14}C]oxoglutarate in malate-loaded mitochondria[39] have shown that fuscin at 25 μM lowers by no more than 10 % the rate of oxoglutarate uptake. It can therefore be concluded that the inhibitory effect of fuscin on the oxidation of the Krebs cycle substrates is either linked to an inhibition of phosphate uptake (which is required directly or indirectly for dicarboxylate or tricarboxylate transport) or to a reaction of fuscin with the lipoamide dehydrogenase system of the α-ketoacids in the Krebs cycle.

DISCUSSION

Data recorded in this paper report evidence that fuscin, a colored quinonoid compound, reacts covalently with –SH groups. Its colour (peak at 365 nm at pH 7.4) disappears when it binds to –SH compounds: its binding can therefore be easily monitored.

The effect of fuscin was tested on a number of mitochondrial functions involving SH groups. Among the reactive mitochondrial components, the respiratory chain when assayed in sonicated mitochondrial fragments was found to be the least sensitive. Fuscin inhibits the first region of this chain, half inhibition requiring about 0.1 mM fuscin as shown with sonicated mitochondrial fragments.

The high reactivity of fuscin with lipoic acid or lipoamide probably accounts for the inhibition of oxidation of the α-ketoacids of the Krebs cycle through an interference with the lipoamide dehydrogenase system.

The inhibitory effect of fuscin on glutamate and ADP transports is interesting in that, among other known SH reagents, only *N*-ethylmaleimide exhibits a similar inhibitory effect on these transports. For instance, other thiol combining reagents like mersalyl and PCMB although being potent inhibitors of the phosphate transport in mitochondria[34,40] are much less efficient than fuscin and *N*-ethylmaleimide on glutamate and ADP transports. If the lipophilic nature which distinguishes fuscin and *N*-ethylmaleimide from other SH reagents underlies these specific effects on anion transport, one may wonder to what extent accessibility to the matrix space can explain these differences. It is shown here (Table II) and in the accompanying paper[18], that avenaciolide, considered up to now as a specific inhibitor of glutamate transport due to its steric analogy with glutamate[17] shares with fuscin and *N*-ethylmaleimide the property of being a penetrant lipophilic SH reagent.

The effect of fuscin on the ADP transport deserves some comments. This is a more complicated phenomenon than the action on glutamate transport Mitochondria must be preincubated not only with fuscin but with a micromolar amount of ADP (or ATP) in order that ADP transport be blocked. This may be explained by the unmasking of mitochondrial –SH groups upon preincubation with ADP (or ATP). These –SH groups appear to play a critical role in the functioning of the ADP carrier. The specificity of ADP or ATP in bringing this effect strongly suggests that preincubated nucleotides bind with the ADP carrier and that the unmasking of –SH group(s) is a result of this binding. Clearly, reactivity of functional –SH groups is influenced by many factors affecting membrane conformation which are superimposed on reagent properties of solubility and structure

Finally fuscin inhibits phosphate uptake. The effect of fuscin on phosphate transport is less marked than that of mersalyl. This may be due to the fact that fuscin, like *N*-ethylmaleimide[41] inhibits only the phosphate–hydroxyl exchange while mersalyl inhibits both the phosphate–OH^- and the phosphate–dicarboxylate carrier[41] It may partly explain the inhibition by fuscin of the oxidation of Krebs cycle anions since phosphate is required for the transport of malate and succinate and that in turn dicarboxylates are exchanged for tricarboxylates and oxoglutarate[42].

Fuscin has been used, in this study, in a range of concentration which did not allow to titrate all the thiol content of rat liver mitochondria estimated to be 85–100 nmoles per mg of protein by Riley and Lehninger[19] and Hadler *et al.*[23]. The membrane functions which are impaired first by fuscin are linked to substrate–anion transport. The –SH groups most reactive with fuscin are located in the matrix space. The high affinity sites for fuscin ($K_d \approx 1\ \mu M$) are linked to matrix components which are lost by sonication of mitochondria. The amount of high affinity sites correlates with the glutathione content of mitochondria (Table I) and actually a preincubation with fuscin lowers the content of free glutathione (Table II) without altering the morphology of the particles as judged by electron microscope. This is a proof that fuscin is able to enter the matrix space of mitochondria. The same statement holds for *N*-ethylmaleimide which was shown to be present in the matrix space as glutathione–*N*-[^{14}C]-ethylmaleimide when *N*-[^{14}C]ethylmaleimide was used. Although the redox potential of fuscin ($E_m = 0.107$ V at pH 7.9 (ref 7)) should enable it to oxidize reduced

glutathione, glutathione seems to form preferentially addition derivatives with fuscin; several lines of evidence favor this statement; (1) the amount of intramitochondrial GSSG is not increased after incubation of mitochondria with fuscin (Table II), (2) fuscin adducts with –SH compounds have been isolated[2,3], (3) a glutathione conjugate has been isolated by thin-layer chromatography (this paper). In the same conditions 2,6-dichloroindophenol also enters mitochondria but oxidizes a large part of internal glutathione. The accessibility of fuscin, *N*-ethylmaleimide and 2,6-dichloroindophenol to the matrix space correlates with their lipophilic nature in contrast with other –SH reagents such as mersalyl, PCMB or DTNB which are probably not readily penetrant.

Along with (ref. 23) one may speculate whether glutathione has a role in protecting sensitive –SH groups located on the matrix side of the inner membrane. In this case, the differing inhibitory effects of penetrant and non-penetrant –SH reagents on transport of glutamate and phosphate may reflect in some measure a differing accessibility of the sensitive loci of their respective translocators with respect to inner and outer faces of the membrane. This suggestion must, however, be made tentatively in view of the many other factors relating to the stereochemistry of the reagent and its substrate.

Glutathione in the mitochondrial matrix may be a reservoir of reducing equivalents capable of buffering or preventing the effects of oxidants on sensitive –SH groups belonging for instance to lipoamide dehydrogenase, anion permeases or respiratory chain and located on the matrix face of the inner membrane. In that manner it can be understood that fuscin entering mitochondria first titrates free glutathione before reacting with less accessible –SH groups in the inner membrane. Comparative experiments with diamide[43] which like 2,6-dichloroindophenol[21–23] is able to oxidize glutathione and with 6,6′-dithionicotinic acid[44] able to react with mitochondrial –SH components, would be of interest.

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