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THE ACCEPTOR SPECIFICITY OF FLAVINS AND FLAVOPROTEINS

II. FREE FLAVINS

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SUMMARY

1. For comparison with flavoprotein oxidases, a study has been made of free flavins in the reduced form with respect to the specificity and stoichiometry of their oxidation by a series of acceptors.

2. Reduced flavins uncombined with proteins show very little acceptor specificity and react very rapidly with nearly all the commonly used acceptors. Their behaviour resembles that of dithionite very closely indeed, and it differs considerably from that of flavoproteins. Like dithionite, free reduced flavins reduce O_2 quantitatively to H_2O_2; this oxidizes a further molecule of flavin.

3. H_2O_2 and cytochrome *c* react more slowly than most acceptors with reduced flavins. Nitrate and NAD^+ do not act at all and require special activation.

4. Catalase can act as a catalyst for the aerobic oxidation of flavins by converting slowly-reacting H_2O_2 into rapidly-reacting O_2.

5. In the absence of catalytic metals ascorbate reacts with acceptors much more slowly than reduced flavins do.

INTRODUCTION

Flavoprotein enzymes differ markedly from one another with respect to the reactivity of their flavin groups towards acceptors. Since in the majority of cases the same flavin group is involved, such differences must be due to the effect on it of combination with the different proteins (apoenzymes). In order to study this effect it is necessary to compare the acceptor specificity of the flavoproteins with that of the free flavins in the absence of proteins. The present paper describes the behaviour of free flavins in the reduced form towards a variety of commonly used acceptors, and compares this behaviour with that of a fairly general chemical reducer (dithionite) and a non-enzymic biological reducer (ascorbate).

METHOD

Since free reduced flavins are very rapidly oxidized by O_2, it is necessary to work under strictly anaerobic conditions. The technique for anaerobic spectrophotometry described in the preceding paper[1] (Part I) was used throughout.

Abbreviation: DCIP, dichlorophenolindophenol.

The general procedure was as follows. Oxidized flavin (FAD or FMN) and phosphate buffer were placed in the cuvette and deaerated as previously described. The flavin was then reduced by adding dithionite, avoiding excess. Finally the acceptor was added, and the course of its reduction by the flavin was followed by recording either the disappearance of the acceptor spectrum or the reappearance of the spectrum of oxidized flavin. In this procedure it is absolutely essential to avoid any excess of dithionite, in order to ensure that the reduction of the acceptor is due to the reduced flavin and not to residual traces of dithionite. For this reason only enough dithionite is added to reduce the flavin partially. The reaction between flavin and dithionite is so rapid that so long as oxidized flavin remains there can be no trace of dithionite left. This can be confirmed by the absence of the dithionite band at 314 nm.

At the moment when the acceptor is added the solution contains only reduced and oxidized flavin, sulphite and buffer. It was shown in Part I (ref. 1) that the flavin is not reduced by sulphite. The majority of acceptors are either not reduced by it, or are reduced so slowly that there is no difficulty in distinguishing this reaction from the reduction of the acceptor by the reduced flavin (see Table II). Only in one case (benzoquinone) is there any real difficulty, as shown below.

In Table I are collected the wavelengths and molar absorbances used in the present work.

TABLE I

MOLAR ABSORBANCES USED

The A_m is the absorbance of a 1 M solution of the substance in a 1-cm cuvette. The wavelengths given correspond to the maxima of absorption bands, except for methylene blue, reduced phenazine methosulphate, ferri- and ferrocyanide at 314 nm, and oxidized cytochrome *c*.

Substance	*Wavelength (nm)*	*Molar absorbance (A_m)*		*Ref.*
		Reduced form	*Oxidized form*	
FMN	450		12 200	2
FAD	450		11 300	2
NAD	340	6220	0	3
DCIP	600	0	21 000	4
Methylene blue at isosbestic point	610	0	41 000	5; present work
Phenazine methosulphate	388	1500	22 000	6; present work
Benzo(hydro)quinone	295	2640	322	Present work
Ascorbate	265	15100		Present work
Dithionite	314	8000	0	Present work
Ferri(ferro)cyanide	420	0	1020	8
Ferri(ferro)cyanide	314	340	1140	Present work
Cytochrome *c*	550	29500	8300	7

RESULTS

Reaction of reduced flavins with acceptors

The behaviour of the two flavin nucleotides (FAD and FMN) is very similar, and as no significant differences between them were found, it will not be necessary to give experimental results for both. Most of the results given are for FAD. Riboflavin was not used.

Ferricyanide

A typical experiment is shown at A in Fig. 1. The absorbance at 450 nm gives the concentration of oxidized FAD present. Initially the system contained $2.5 \cdot 0.81/11.3 = 0.18$ μmole of FAD. After the addition of dithionite 0.18—$2.51 \cdot 0.44/11.3 =$ 0.082 μmole of reduced FAD were present, corresponding to the 0 082 μmole of dithionite added. On adding ferricyanide, the reoxidation of the flavin was almost instantaneous*. The amount of ferricyanide added was insufficient to oxidize all the reduced flavin, as it was desired to determine the stoichiometry of the reaction. The amount of reduced FAD oxidized by the 0.115 μmole of ferricyanide was $(2.52 \cdot 0.70 - 2.51 \cdot 0.44)/11.3 = 0.058$ μmole, so that 2 molecules of ferricyanide oxidize 1 molecule of reduced flavin.

Oxygen

Reduced flavins react extremely rapidly with dissolved O_2. The reaction with O_2 bubbled through the solution is much less rapid, because it is limited by the rate at which O_2 dissolves in water, which is a relatively slow process.

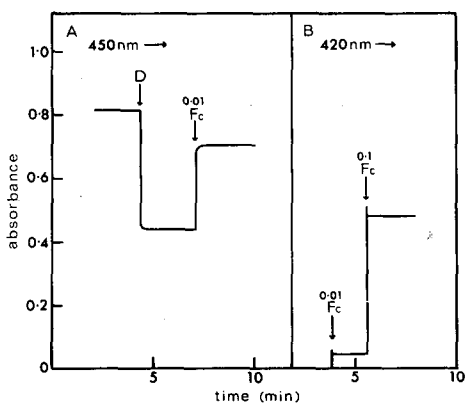


Fig. 1. Oxidation of reduced FAD by ferricyanide. A. Initially, 2.4 ml 0.1 M phosphate buffer (pH 7.6) + 0.1 ml 1.8 mM FAD. At point D, 0.01 ml 8.2 mM dithionite, and at Fc 0.01 ml 11.0 mM ferricyanide added. B is a check on concentration of the ferricyanide solution. Initially, 2.5 ml phosphate buffer (pH 7.6). At points Fc, 0.01 ml and 0.1 ml ferricyanide solution added.

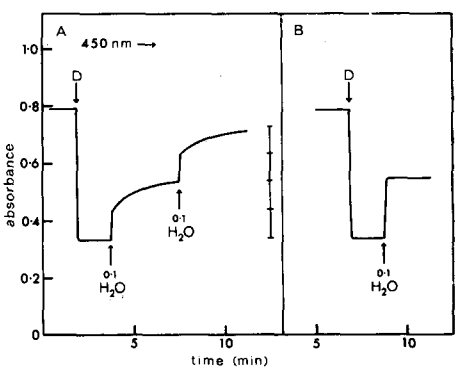


Fig. 2. Oxidation of reduced FAD by O_2. A. Initially, 2.0 ml 0.1 M phosphate buffer (pH 7.6) + 0.5 ml 0.344 mM FAD. At D, 0.05 ml 1.94 mM dithionite added, and at second and third arrows 0.1 ml water saturated with air at 22°. (0.1 ml water contained 0.0275 μmole O_2.) B. As A, but initially with 1.5 ml phosphate + 0.5 ml FAD + 0.5 ml catalase solution.

Fig. 2A, shows the oxidation of reduced FAD on addition of small amounts of air-saturated water. Each division of the scale on the right corresponds to the oxidation of 1 molecule of flavin per molecule of O_2 added. The reaction occurs in two phases, namely a very rapid oxidation of 1 molecule followed by a much slower oxidation of a second molecule, thus resembling the oxidation of dithionite by O_2 (Fig. 9 of the preceding paper[1]). It has long been known that flavoprotein oxidases reduce O_2 to H_2O_2, and it is evident that the second molecule of FAD was oxidized

* GIBSON AND HASTINGS[9] state that the oxidation of reduced FMN by ferricyanide at pH 6.3 is almost complete within 3 msec, but give no details.

by the H_2O_2 formed during the oxidation of the first. Fig. 3 shows directly that H_2O_2 oxidizes reduced flavin much more slowly than O_2 does.

Fig. 2B is a duplicate of 2A, but with a moderate amount of catalase added initially. The two phases of the reaction are now replaced by a single very rapid step giving an oxidation of 2 molecules of flavin per molecule of O_2. This is an interesting case of catalase acting as an oxidation catalyst by converting the slowly reacting H_2O_2 into the rapidly reacting O_2.

GIBSON AND HASTINGS[9] studied the kinetics of the oxidation of $FMNH_2$ by O_2 at pH 6.3, using rapid reaction techniques. The reaction is less rapid than that with ferricyanide. They observed two phases, resembling those of Fig. 2A, and attributed the slow phase to oxidation by H_2O_2. The kinetics of the fast phase, *i.e.* the oxidation by O_2, were complex, and they state that 'so far, no entirely satisfactory scheme has been developed'. The results suggested that the oxidation proceeded by two parallel paths, (a) by way of an addition compound of $FMNH_2 + O_2$, as already suggested by GUTFREUND[10], and (b) in the later stages of the oxidation by way of a compound of $FMNH_2 + FMN$.

It has recently been suggested[11–13] that in the case of the metal-containing flavoprotein xanthine oxidase (EC 1.2.3.2) the first product of the reduction of O_2 is the peroxide radical anion $\cdot O_2^-$, often called superoxide anion. This radical was also shown by BALLOU *et al.*[14] to be formed in the oxidation of a related compound (reduced tetraacetyl riboflavin) in alkaline solution (pH 10.6), but the amount found was strongly dependent on pH, and in neutral solution the yield was very small. In any case the radical has an extremely short lifetime in the presence of water and yields H_2O_2, so that its formation would make no difference to the present experiments. The question is discussed further in the following paper[15].

Benzoquinone

This is one of the most reactive acceptors; as already mentioned, however, there is a slight complication because it reacts rapidly with the sulphite formed from the

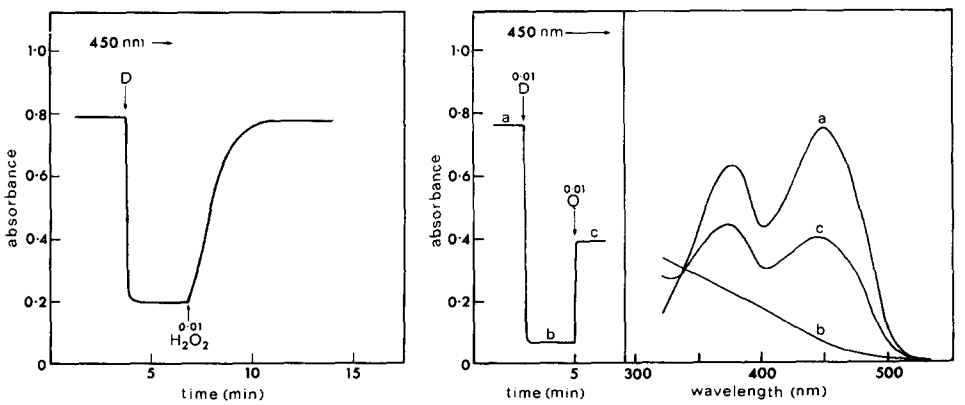


Fig. 3. Oxidation of reduced FMN by H_2O_2. Initially, 2.0 ml 0.1 M phosphate buffer (pH 7.6) + 0.5 ml 0.35 mM FMN. At D, 0.01 ml 13.3 mM dithionite added, and at second arrow 0.01 ml 20 mM H_2O_2 (excess).

Fig. 4. Oxidation of reduced FMN by benzoquinone. Initially, 2.0 ml 0.1 M phosphate buffer (pH 7.6) + 0.5 ml 0.31 mM FMN. At D, 0.01 ml 15 mM dithionite, and at Q 0.01 ml 20 mM benzoquinone added. The spectra were recorded at points a, b and c.

dithionite[1]. Fig. 4 shows an experiment in which just enough dithionite was added to reduce the flavin completely, followed by a slightly larger amount of quinone. Only about half the equivalent amount of reduced flavin was reoxidized, doubtless because slightly more than half the quinone had been used up in oxidizing sulphite formed from the dithionite in the first step.

The absorption spectra of the mixture were recorded at points a, b and c; a shows the spectrum of oxidized FMN and b that of reduced FMN, while c corresponds to approximately half-reduced FMN, lying midway between a and b and showing no evidence of flavin semiquinone formation. From the equilibrium constant given by LOWE AND CLARK[16] only about 2 % of the flavin would be in the semiquinone form under these conditions.

The exceptional reactivity of quinone as an acceptor* is well shown with ascorbate as donor (see Table II).

Methylene blue

This is a classical acceptor, used in much work over the past 40 years; but although it reacts readily with a great many systems, it has two properties which are rather unsatisfactory for spectrophotometric experiments.

The first is that it exists in solution as an equilibrium mixture of monomer and dimer, the ratio changing with the concentration[5,18]. These have different spectra; the monomer has a strong band at 660 nm and the dimer has one about half as strong at 600 nm. Consequently the absorbance at either wavelength is far from being proportional to the concentration. There is, however, an approximately isosbestic point at about 610 nm. This wavelength can therefore be used, but only for rough work, as it is not accurately isosbestic.

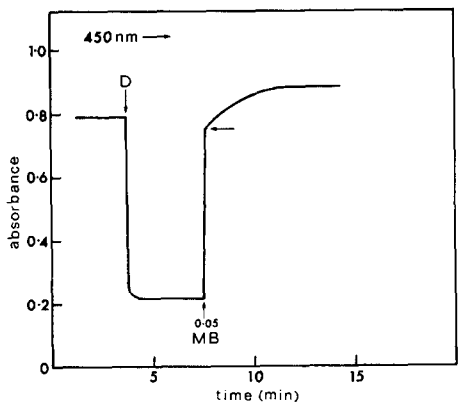
The second property is that the reduced form is insoluble in water, and this gives rise to the kind of effect shown in Fig. 5. Here the reaction between reduced FAD and methylene blue is followed by recording the absorbance of oxidized FAD at 450 nm, at which wavelength the absorbance of methylene blue is very small. The amount of methylene blue added (0.11 μmole, including the trace of O_2 dissolved in it) was slightly less than the reduced flavin (0.13 μmole), and at the horizontal arrow it was completely decolorized and 0.12 μmole of the flavin had been reoxidized. But after this there was a further apparent increase of absorbance to a value well above that corresponding to 100 % oxidation. On recording the spectrum, however, it became clear that the increase was not due to absorbance but to a shift of the base-line by over 0.1 unit due to light scattering, caused by the crystallizing out of reduced dye in the form of a scarcely visible cloud of microscopic needles.

2,4-Dichlorophenolindophenol (DCIP)

This widely used acceptor reacts very rapidly with reduced flavins, and it was shown in the preceding paper[1] to be reduced also by sulphite, though more slowly. The first part of Fig. 6 shows the rapid and quantitative reoxidation of 0.045 μmole of reduced FAD by 0.1 μmole of DCIP, followed at 450 nm, the wavelength of the flavin band. (The slight difference between the absorbances at the beginning and end of this part is simply due to dilution by the reagents added.) The wavelength was then quickly changed to 600 nm, that of the band of DCIP. At this point there is no longer any dithionite or reduced flavin present. B shows the initial absorbance at

* After this work was completed, GIBIAN AND RYND[17] showed that reduced riboflavin is oxidized by quinone in less than 3 msec.

this wavelength, determined in a duplicate experiment with no dithionite added, and b shows the calculated absorbance change produced by the reduction of 0.045 μmole of DCIP, *i.e.* the amount corresponding to the amount a of reduced FAD. The curve then shows the reduction of a second molecule of DCIP by the sulphite formed from the dithionite.



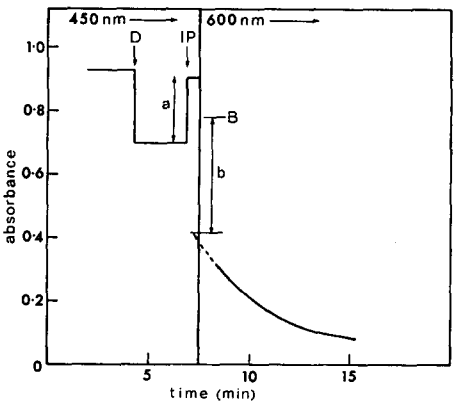


Fig. 5. Oxidation of reduced FAD by methylene blue. Initially, 2.0 ml 0.1 M phosphate buffer (pH 7.6) + 0.5 ml 0.365 mM FAD. At D, 0.1 ml 1.3 mM dithionite, and at second arrow 0.05 m 1.85 mM methylene blue added. At the horizontal arrow there was no blue colour and 1 moleculel of flavin had been oxidized per molecule of methylene blue.

Fig. 6. Oxidation of reduced FAD by DCIP. Initially, 2.0 ml 0.1 M phosphate buffer (pH 7.6) + 0.5 ml 0.41 mM FAD. At D, 0.1 ml 0.45 mM dithionite, and at IP 0.03 ml 3.3 mM DCIP added. After the division the recording was continued at the wavelength of DCIP absorption. B shows the absorbance of a duplicate mixture without dithionite addition. b represents the absorbance of an amount of DCIP equivalent to the amount of flavin shown by a.

Other acceptors

Phenazine methosulphate is the best of the acceptors with certain flavoproteins[19]. With reduced FAD it behaves much like DCIP, but it reacts with sulphite rather more rapidly.

Cytochrome c was shown by SINGER AND KEARNEY[20] to be readily reduced by reduced riboflavin. By the present method it was found that both $FMNH_2$ and $FADH_2$ reduce it noticeably more slowly than they do most other acceptors, but still rather too fast for accurate measurements of the rate.

MASSEY *et al.*[21] have shown very recently that the reduction of cytochrome *c* by a flavin derivative (tetraacetyl riboflavin, reduced by ethylene diamine tetra-acetate in light) is accelerated by the presence of O_2, due to the formation of the $\cdot O_2^-$ radical.

NAD$^+$, though it is reduced very rapidly by a great many dehydrogenases, is reduced comparatively slowly by dithionite, and no reduction by $FADH_2$ could be detected. It is true that NAD^+ lies below flavins on the redox scale, so that the equilibrium of the reaction would be in favour of the reverse direction. Nevertheless on testing the reduction of FAD by NADH, no reaction could be detected by measurements at either 450 nm or 340 nm. Indeed if the coenzyme had been able to react with flavins in the absence of enzymes, the equilibrium constant is not so large as to

prevent observation of a small amount of reduction of NAD^+ by $FADH_2$. It must be concluded that NAD^+ does not react with flavins in the absence of catalysts.

Nitrate has long been known to act as an acceptor with certain flavoprotein enzymes, but no reaction with reduced flavins could be detected. This is discussed further in Part III (ref. 15).

Reaction of acceptors with ascorbate

For comparison with the other reducers, the results obtained with ascorbate are shown in the last line of Table II, In general the reactions of ascorbate are considerably slower than those of dithionite or reduced flavins, although the reaction with benzoquinone is still very rapid. Ascorbate differs from these reducers in two respects. The first is that it has a tendency to be activated as a reducer by traces of catalytic metals, for example with O_2 as acceptor, and the negative result obtained here with O_2 is presumably due to the use of glass-distilled water in making up the solutions. The second difference is that ascorbate lies considerably higher than the others on the redox scale, in fact above the flavins. It could not therefore be expected to reduce FAD, but $FADH_2$ should reduce the oxidized form of ascorbate.

Fig. 7 shows that this reaction occurs, but rather slowly. The oxidized ascorbate was produced by oxidizing ascorbate with ferricyanide. In order to avoid any possibility that the oxidation of flavin was due to residual ferricyanide, insufficient ferricyanide was added to oxidize all the ascorbate, and the oxidation was monitored at 265 nm until some min after it had terminated. The solution added to the reduced FAD at the point OA in Fig. 7 therefore contained, in addition to oxidized ascorbate, some ascorbate and ferrocyanide, but no ferricyanide.

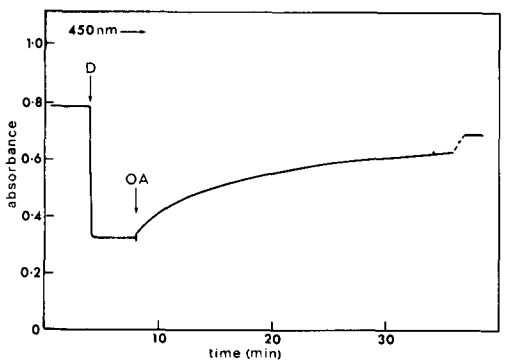


Fig. 7. Oxidation of reduced FAD by oxidized ascorbate. Initially, 2.0 ml 0.1 M phosphate buffer (pH 7.6) + 0.5 ml 0.35 mM FAD. At point D, 0.04 ml 2.5 mM dithionite, and at OA 0.05 ml 2.5 mM oxidized ascorbate added. Oxidized ascorbate prepared by mixing equal volumes of 10 mM ascorbate and 10 mM ferricyanide and incubating until absorbance at 265 nm constant.

The fact that the reduction of cytochrome *c* by ascorbate is so slow in the absence of enzymes (Fig. 8) is of some interest, because ascorbate has often been used as a substrate in testing the cytochrome oxidase system of heart muscle[22,23], where the reaction must take place rapidly. This suggests that the muscle preparations contain some factor catalysing the reaction of ascorbate with cytochrome *c*.

TABLE II

SUMMARY OF RESULTS

VR indicates very rapid (half-time less than 5 sec), R rapid (10–20 sec), M moderate (30 sec–4 min), S slow (5–20 min), VS very slow (greater than 40 min). o indicates no reaction detected. The half-times and the molecular ratios are given were possible. PMS = phenazine methosulphate.

Reducers	*Acceptors*										
	Oxygen	*H_2O_2*	*Ferri-cyanide*	*FAD*	*Benzo-quinone*	*Cyt. c*	*NAD^+*	*DCIP*	*Methylene blue*	*PMS*	*Nitrate*
$FADH_2$	VR 1:1	M 1:1	VR 1:2	—	VR 1:1	R 1:2 15 sec	o	VR 1:1	VR 1:1	VR 1:1	o
Dithionite	VR 1:1	M 1:1 2 min	VR 1:2	VR 1:1	VR	VR 1:2	S 1:1 5 min	VR 1:1	VR	VR 1:1	o
Sulphite	VS	VS 50 min	VS 1 h	o	VR 1:1	o	o	M 1:1 2 min	o	M 1:1 75 sec	
Ascorbate	o	S 20 min	M 1:2 30 sec	o	VR 1:1	S 1:2 10 min	o	M 1:1 30 sec	VS 90 min		o

MINNAERT[23] observed a much more rapid reduction of cytochrome *c* by ascorbate, but this was probably due to catalysis by the traces of heavy metals which he states were present in the reagents.

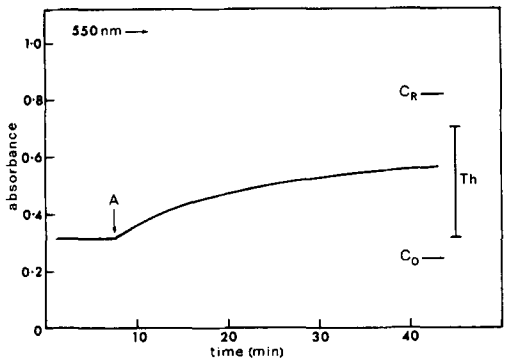


Fig. 8. Reduction of cytochrome *c* by ascorbate. Initially, 2.5 ml 0.1 M phosphate buffer (pH 7.6) + 0.05 ml 1.5 mM cytochrome *c* (87.5% oxidized). At point A, 0.01 ml 2.5 mM ascorbate added. C_R and C_O correspond to fully reduced and fully oxidized cytochrome. Th shows theoretical absorbance change for reduction of 2 molecules of cytochrome per molecule of ascorbate.

DISCUSSION

Table II gives a summary of the results in the form of a comparative table, in which the rates of the reactions are shown on a semi-quantitative scale from 'very rapid' (VR) to 'very slow' (VS). As the aim of the present work was merely to get a general picture of the acceptor specificity of reduced flavins in comparison with other reducers and with flavoproteins, there was no need for measurements of the velocity constants of the reactions, which would have required much more elaborate apparatus, but where possible the time taken for the reaction to proceed half-way is given. This, however, is only a rough guide, as the concentrations varied in the different experiments. The stoichiometry is shown wherever determined, '1:2' denoting that 1 molecule of the reducer reduces 2 molecules of the acceptor. These figures relate only to the direct reaction, and ignore any subsequent reaction between products of the first stage.

It is evident from the table that the reduced flavins show very little specificity towards acceptors and they resemble dithionite very closely indeed in their reducing properties. They are oxidized very rapidly by most of the acceptors, but more slowly by H_2O_2 and by cytochrome *c*. NAD^+ and nitrate do not act at all, and evidently require special activation in order to act as acceptors with flavoproteins.

In general, ascorbate is a much slower reducer in the absence of catalytic metals, but outstanding here is the very rapid reaction with quinone.

In the following paper[15] it will be shown that the behaviour of free flavins towards acceptors is entirely different from the behaviour of the flavin groups when present in flavoproteins.

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