BBA 46070

THE ACCEPTOR SPECIFICITY OF FLAVINS AND FLAVOPROTEINS

I. TECHNIQUES FOR ANAEROBIC SPECTROPHOTOMETRY

MALCOLM DIXON

Department of Biochemistry, University of Cambridge, Cambridge (Great Britain)

(Received September 10th, 1970)

SUMMARY

1. Easily constructed apparatus is described for spectrophotometry under strictly anaerobic conditions without requiring special cuvettes. It permits the addition of several reagents successively without opening the system to the air.

2. The absorption spectrum of dithionite shows a strong peak at 314 nm, the molar absorbance of which has been determined. This gives a convenient method for the titration of acceptors with dithionite.

3. One molecule of dithionite reacts very rapidly with one molecule of O_2 in solution. The O_2 is reduced quantitatively to H_2O_2. With excess of dithionite another, much slower, reaction follows, in which a second molecule of dithionite is oxidized by the peroxide.

4. A study has been made of the reduction by dithionite of a variety of acceptors commonly used in the study of flavoproteins. The majority react very rapidly, but a few are reduced relatively slowly or not at all.

5. The majority of acceptors do not react significantly with sulphite, the oxidation product of dithionite. One molecule of dithionite then provides two reduction equivalents. A few acceptors, however, react with the sulphite formed, giving a second reaction involving two more equivalents.

INTRODUCTION

It appears that every flavoprotein enzyme acts by a mechanism that involves a cycle of reduction of its flavin group by one reactant (the substrate) and its reoxidation by another (the acceptor). The specificity towards the substrate identifies the particular enzyme; the specificity towards the acceptor determines the sub-group to which the enzyme belongs, for example whether it is classed as an oxidase or a cytochrome reductase or a dehydrogenase.

Extensive studies have been made of the substrate specificities of flavoproteins, which are usually found to be rather high, only one substance or a limited group of closely related substances being capable of acting as substrate. The specificity towards acceptors, on the other hand, has been much less studied; and because in many cases

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulphate; A_M, molar absorbance.

several chemically unrelated substances have been found to act, it seems to have been tacitly assumed that the acceptor specificity of flavoproteins is low and therefore relatively uninteresting. The literature, however, contains sufficient data to show that there are quite sharp and sometimes dramatic differences between the abilities of different flavoproteins to react with particular acceptors.

In the majority of cases it is the same flavin that is being oxidized; differences in specificity of reaction towards acceptors must therefore be conferred on the flavin by combination with the different enzyme proteins (apoenzymes).

In order to see the nature of the effect of the proteins on the reactivity of the flavin, it is necessary to compare the behaviour of the reduced flavoproteins towards acceptors with that of the free reduced flavins in the absence of proteins. The information in the literature on the acceptor specificity of free flavins is rather meagre, so that it is first necessary to study the non-enzymic oxidation of free reduced flavins by a sufficient number of acceptors of different types to get a general picture of the reactivity. The fact that this has not been done to any great extent previously, in spite of the suitability of recording spectrophotometers for the purpose, is no doubt due to the very rapid reaction between reduced flavins and oxygen, which makes it absolutely necessary to maintain stringent anaerobic conditions throughout each experiment.

The usual methods for working in anaerobic conditions were found not to give a sufficient degree of freedom from O_2 for this work, and it was only after many experiments that a satisfactory technique was devised. As this technique has proved useful for a number of purposes, and the apparatus is easy to set up without requiring special expensive cuvettes, it is described here in some detail.

This paper (Part I of the study) therefore deals with the technique of anaerobic spectrophotometry and with the use of dithionite as a reducer in quantitative experiments; Part II is concerned with the acceptor specificity of free flavins, and Part III with the acceptor specificity of flavoproteins.

TECHNIQUE FOR ANAEROBIC SPECTROPHOTOMETRY

Principles

There are two distinct methods for removing the O_2 from the cuvette used in the spectrophotometer, namely (a) the use of a closed system with initial removal of air by evacuation, and (b) the displacement of the air by passing a continuous stream of O_2 free gas (usually N_2) through the cuvette during the experiment.

The vacuum method is usually carried out in a Thunberg–Beckman cell of the type illustrated by DIXON AND KENWORTHY[1], first used many years ago (but not illustrated) by Keilin and Hartree and others*. After the appropriate solutions are measured into the cell, the greased stopper is placed in position, the cell is evacuated by attachment to a pump and the system is closed by turning the stopper. The reaction is usually started by tipping in one of the reagents from the hollow stopper, and it then takes place in a vacuum.

This method has certain disadvantages:

(i) It is not easy to attain sufficient freedom from O_2 for work such as the present. There is no difficulty in removing the air from the gas space, as it is swept out by the

* After the present work was completed, two more sophisticated forms of cuvette were described by FOUST *et al.*[2] and BURLEIGH *et al.*[3], both for method (a).

stream of water vapour when the vacuum is applied. It is not sufficiently realized, however, that the dissolved O_2 comes out relatively slowly, and some may not have been removed by the time when the cell is closed off.

If, for example, 4 ml of air-saturated solution are taken, there will be initially about 25 μl of dissolved O_2. But in studying flavins and acceptors we are dealing with substances whose molar absorbances are often of the order of 10000, and as it desirable for technical reasons to aim at an absorbance of 0.5 to 1.0, it follows that there may be only 0.2 μmole of flavin present in the cell. If errors due to residual O_2 are to be kept below 1 %, the amount of O_2 in the cell must be below 0.002 μmole or 0.04 μl, which is about 1/500 part of the amount initially dissolved.

(ii) In many experiments it is desirable to be able to add several reagents in succession without introducing air. Since the Thunberg–Beckman cell is a closed system this cannot be done, although DIXON AND KENWORTHY[1] used a device by which two solutions can be added successively. The very recently introduced cuvette of BURLEIGH *et al.*[3], however, has a special gas-locked rubber septum which can be pierced by the needle of a syringe for the injection of solutions.

(iii) In the vacuum-cell method it is necessary to remove the cell temporarily from the spectrophotometer in order to add a reagent and mix it with the main solution. Consequently the part of the reaction curve immediately after the addition is missing from the record.

(iv) There are a few minor technical objections to the method, for example the possibility of the liquid shooting up into the stopper during the evacuation, contact of the liquid with the tap grease during the tipping, and changes of volume and concentration due to evaporation of water *in vacuo*.

For these reasons the method was abandoned in favour of the less-used method of passing nitrogen through the cell. This method also has difficulties, the chief of which, apart from purely mechanical questions, are (a) the supply of gas sufficiently free from O_2, (b) the addition of reagents during the experiment without opening the cuvette, and (c) the avoidance of introduction of O_2 with the reagents added.

When a stream of gas is continuously passed through the cuvette, the requirement of O_2 freedom of the gas is especially stringent because during the course of a long experiment a comparatively large volume of the gas may pass through. Under the conditions finally adopted, about 1 l of N_2 will pass through in the course of an hour's experiment and if, as mentioned above, the total amount of O_2 introduced is not to exceed 0.04 μl, the N_2 must contain less than 1 part of O_2 in $25 \cdot 10^6$. In practice, it is true, the O_2 is not likely to react rapidly at such a low concentration, so that somewhat less stringent conditions may give satisfactory results, but every effort must be made to eliminate traces of O_2. The method described below has been found to give a satisfactory degree of anaerobiosis.

The addition of reagents is achieved by having an outlet tube for the gas with a bore that is narrow enough to cause the gas to pass out with sufficient velocity to prevent any external air from penetrating back into the cell. The reagents are then added from a Hamilton micro-syringe with a fine needle that can be passed down this outlet tube. The necessity of deoxygenating all the reagents before addition is avoided by adding them in very small volumes, so that the amount of dissolved O_2 added is negligible, *e.g.* 0.01 ml of an air-saturated solution would contain about 0.05 μl of O_2.

Apparatus

(a) O_2-free N_2

The essential requirements are a supply of commercial N_2 with a low O_2 content, a means of removing such O_2 as it contains, tubing of material impermeable to O_2 for conducting the gas to the cell compartment of the spectrophotometer, and a special stopper for the cuvette to allow the gas to be passed through it. It is also convenient to include in the outfit an apparatus for the storage of a supply of dithionite solution under anaerobic conditions for use as a reducing reagent.

Ordinary commercial cylinder ('tank') N_2 of course contains far too much O_2 for the purpose. Commercial 'O_2-free' or 'white spot' N_2 contains too much for direct use, but is suitable for supplying the de-oxygenator (D in Fig. 1). This is a 'Nilox' (manufactured by Jencons (Scientific) Ltd., Mark Road, Hemel Hempstead, Hertfordshire, England), which is a 12-ft glass tube coiled inside a 500-ml vessel containing O_2-absorbing solution and designed so that both gas bubbles and liquid pass slowly along the tube. This gives very efficient exposure of the gas to the liquid, an alkaline solution of dithionite.

In filling the de-oxygenator, 500 ml of 0.2 M Na_2HPO_4 are added, the outlet stopper is inserted and N_2 is passed through for about 30 min; the stopper is then momentarily removed, 16 g of solid $Na_2S_2O_4$ (Harrington and Co.) quickly tipped in from a test tube, the stopper replaced and a slow stream of N_2 continued until the dithionite is dissolved. One filling is usually sufficient for 2 or 3 weeks of work under normal conditions. FIESER's[4] solution (dithionite *plus* sodium anthraquinone sulphonate catalyst) may be still more effective but was not used, as dithionite was found to be adequate for the present work. With some samples of dithionite a slight odour may be noticed at first in the emergent gas, but this soon passes off and has not been found to affect the results.

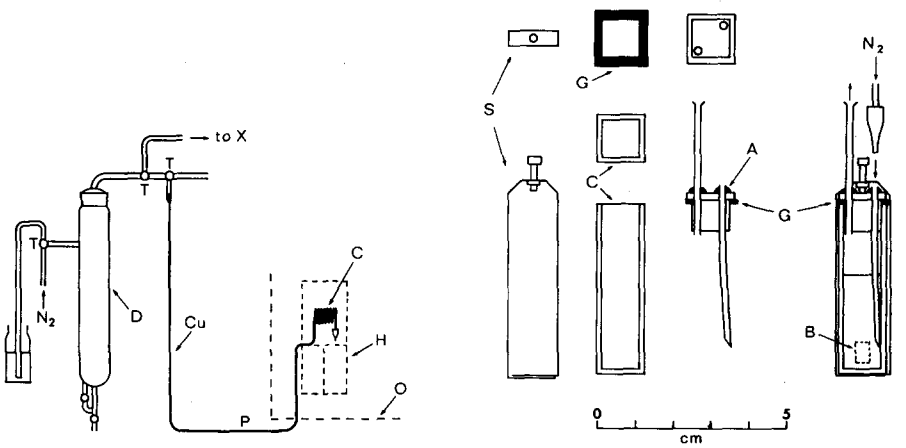

Fig. 1. Arrangement for supply of O_2-free N_2. D, deoxygenator; T, T-taps; Cu, copper tubing; C, coil to give elasticity; O, outer casing of Beckman DB spectrophotometer; H, cuvette holder of spectrophotometer.

Fig. 2. Arrangement for passing nitrogen through cuvette. C, Beckman 10-mm cuvette; S, thin brass strap with screw for clamping perspex stopper firmly in cuvette; G, rubber gasket fitting stopper; A, araldite epoxy-resin sealing polythene tubes in stopper; B, position of light beam through cuvette. For details see text.

(*b*) *Spectrophotometric arrangements*

The gas is conducted to the spectrophotometer through soft-drawn copper tubing of 1/16-inch outside diameter (Cu in Fig. 1) (obtainable from K. R. Whiston, New Mills, Stockport, Cheshire, England). No rubber or plastic tubes tried were sufficiently impermeable to O_2 (see Fig. 4). No trace of contamination of the solutions with copper was ever detected. The end of the copper tube is carefully sealed to the glass side-tube of the T-tap shown, using Apiezon W wax (Edwards High Vacuum Ltd., Manor Royal, Crawley, Sussex, England), the side-tube being previously drawn out to fit the copper tube loosely. The other end is sealed to a short length of thick-walled glass tubing drawn out to form a jet which plugs into the inlet tube of the cuvette stopper (Fig. 2). Thick-walled tubing is used to give strength to the point of the jet, which is otherwise somewhat fragile.

A Beckman DB spectrophotometer with linear-log recorder was used for all the work. It is especially convenient because no modification whatever is necessary. The copper tube is taken through the opening in the bottom of the outer case of the instrument (O in Fig. 1) and into the cell chamber through the sponge-rubber gasket between the top of the cell holder (H) and the cover, being tied firmly to the inlet and outlet tubes provided for the thermostating water. The tube is coiled into a helix of 12 turns of 3/4-inch diameter (C) positioned within the cell chamber, in order to give elasticity, and this is adjusted so that the jet fits naturally with a slight downward pressure into the inlet tube of the cuvette in the cell holder.

Fig. 2 gives a scale drawing of the cuvette and stopper. The cuvette itself (C) is an ordinary 10-mm Beckman quartz cell. It is provided with a perspex stopper, machined to be a comfortable sliding fit, which carries a thin rubber gasket G. A number of such stoppers can be conveniently made at one time from a piece of 1/2-inch perspex sheet on a milling machine with an 1/8-inch cutter. A series of parallel grooves about 9 mm deep are cut with the required spacing and then, without altering the depth of cut a second series of grooves is cut at right angles to the first. This method ensures that the lower surface of the flange that carries the gasket is accurately planar; this is essential. The stoppers are separated by a central cut along the bottom of each groove, preferably with a band-saw, giving a flange of 12.5 mm^2.

The gaskets are cut from red rubber sheet about 1 mm thick, such as the inner tube of a bicycle tyre, to fit the stoppers accurately. The cutting is best done with a piece of safety razor blade broken off to the correct length and held in a hand-vice, the rubber sheet being laid on a flat wooden surface and the blade pushed straight through without any sawing motion. It is advisable to examine each gasket with a lens to make sure that it fits closely all round, without any slits at the corners, which might cause leaks. Each gasket is very slightly greased with a trace of vaseline petroleum jelly before use.

The stopper is held firmly in the cuvette by a strap (S) about 11 mm wide, cut from 0.005-inch brass shim, bent as shown and soldered where it overlaps at the bottom. It is drilled at the top to take an 8 BA cheese-headed brass screw, working in a nut soldered to the strap. When this is screwed down finger-tight (never with a screwdriver) onto the stopper, it prevents the slightest movement of the latter, *e.g.* during the addition of solutions, which would otherwise draw in enough air to vitiate the experiment. The strap is so thin that no alteration to the cell holder is necessary.

The stopper is drilled near diagonally opposite corners to fit closely the gas

inlet and outlet tubes, which are of polythene tubing 1.6-mm outside diameter (No. 2 Sterivac cannula tubing, Allen and Hanbury). The tubes are cemented in place with araldite epoxy-resin, as shown at A. In fact the araldite does not bind onto the polythene, but it holds it very firmly while still allowing it to be moved for adjustment by the exercise of considerable force. After adjustment the inlet tube is cut off level with the top of the araldite, giving a firm socket to receive the glass tip of the nitrogen supply tube; this is very slightly greased with vaseline before it is inserted. The polythene tubing usually has a slight natural curvature which is used to keep the lower end against the side of the cuvette, so that the gas bubbles do not come into the light beam, which is shown at B. The cuvette stands on a 9-mm block placed in the holder, by which it is raised so that the beam passes near (but not grazing) its bottom.

The outlet tube, as already mentioned, is also used for the addition of reagents by means of a Hamilton syringe (No. 710, total capacity 0.1 ml, graduated in μl, with a needle 52 mm long and 0.7 mm outside diameter). It is convenient to cut off the gas outlet to about 25 mm, so that when the syringe is pushed home the tip of its needle just touches the surface of the liquid in the cuvette.

The gas flow was about 15 ml/min. As the internal cross-section of the outlet tube is about 1 mm^2, the gas passed out through it at a speed of about 30 cm/sec, which was ample to prevent any back diffusion of O_2 into the system. At this rate there was no unsteadiness of the recorder pen due to gas bubbles touching the light beam. No stirrer was needed; the passage of gas gave essentially complete mixing of added reagents within 10 sec, which was adequate for the present purpose, especially as none of the recording was lost during the additions. As the recorder was left running, it was convenient to shade the spectrophotometer to some extent, to avoid large movements of the pen when the lid of the cell chamber was opened.

Frothing normally gave no trouble, and even in the few cases where it was observed no appreciable volume of liquid was lost thereby. In these cases, however, a small piece of filter paper with a slit was slipped over the outlet tube to protect the optical faces of the cuvette from any droplets that might be ejected.

The reference cell contained water in all experiments and the temperature was 20°. Glass-distilled water was used in making up solutions.

(*c*) *Stock dithionite solution*

For work of this type a supply of dithionite solution is needed. Contrary to popular belief, dithionite at pH 7.6 and in the absence of O_2 is reasonably stable, at any rate for a period of weeks. (Ref. 3, which appeared after this work was completed, states that dithionite solution at pH 9 is relatively stable for at least 3 days.) Its stability, however, is markedly affected by pH. At pH 7.0 some instability can be detected at room temperature, and there is a rapid breakdown in acid solutions, the rate of loss being about 2 % per min at pH 6.5 and 10 % per min at pH 6.0. Unbuffered dithionite rapidly becomes acid when oxygen is present. In making up dithionite solutions, therefore, it is essential to remove oxygen before the solid is dissolved and to ensure that there is adequate buffering at pH 7.6 or higher.

This may conveniently be done in the apparatus shown in Fig. 3, which consists of a 1-ml graduated burette connected with a vessel closed with a bung of artificial rubber. Dry dithionite is weighed out in the small glass vessel V, which fits loosely within the ring of glass rod R mounted on the lower side of the bung. V rests upon the lever L, which consists of a glass rod bent into three parts mutually at right angles.

(The top section should be imagined as projecting forwards at right angles to the paper, and is a handle by which the lever may be turned).

Buffer (usually 50 ml of 0.2 M phosphate, pH 7.6) is measured into the main vessel, which is then closed with the bung, and by turning the T-taps, first briefly into Position a and then into Position b, a stream of N_2 is passed down through the burette

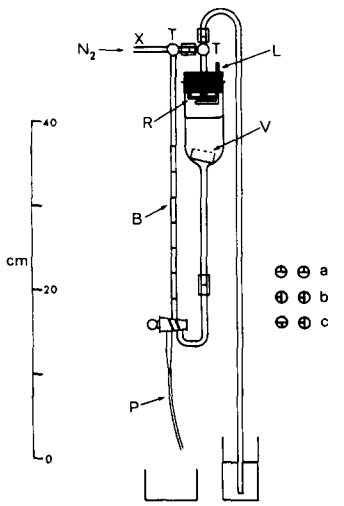

Fig. 3. Arrangement for supply of dithionite solution. B, 1-ml burette; T, T-taps; P, polythene tube waxed to burette tip; V, glass weighing vessel for dithionite; R, ring of glass rod to hold V; L, glass rod lever to support V in R; a, b, c, positions of T-taps.

and up through the buffer in the main vessel until the buffer is thoroughly deoxygenated. This takes at least half an hour, after which the lever L is turned so that V falls into the buffer solution and the dithionite dissolves. The gassing is continued for a few minutes after solution is complete, and then on turning the taps into Position c the dithionite solution flows into the burette. The graduations of the burette were not used in the present work (the apparatus was constructed for another purpose many years ago), but a piece of fine polythene tube (P) was sealed onto the tip for the purpose of filling the Hamilton syringe. The needle of the syringe is passed well up into this tube and the dithionite solution from the burette is allowed to flow past it and strip from the end of the tube while the syringe is filled by steadily withdrawing the plunger. In this way the solution is completely protected from oxygen during transfer to the syringe. The orifice of the needle is so minute that no detectable oxidation occurs during the subsequent transfer of the syringe to the cuvette. It is essential to make sure that no small air bubbles are trapped in the syringe, and it is best to expel the first filling and to refill before use.

The rubber bung of the apparatus should be well-fitting and should be greased lightly with vaseline before insertion. Joints are provided where marked to give the necessary flexibility for inserting and removing the bung; for these, short lengths of tygon tubing are used, within which the glass tubes butt closely together. It is advisable to cover the main vessel with aluminium foil to screen off light. While not in use the taps are kept in Position a. Before use gas is passed briefly through the upper

tubes to remove any traces of air; then the taps are turned into Position c and a slow stream of gas is maintained during use. With these precautions, the solution can be kept for two or three weeks, although there is usually a slow falling off in reducing power (about 10% in 2 weeks), due probably to a small inherent instability. This, however, is of little consequence, as the dithionite concentration is very easily determined when necessary by absorbance measurements, and in any case is usually given by the recording.

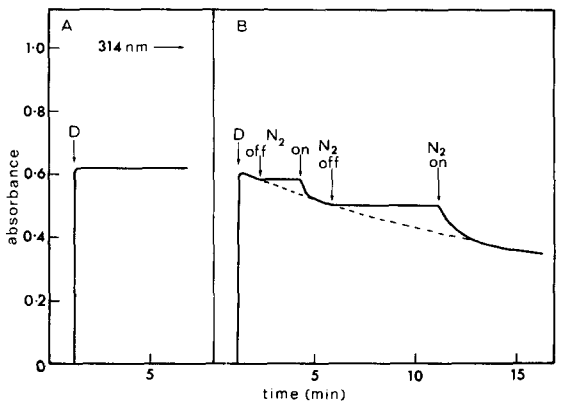

Fig. 4. Test for freedom from oxygen. A. Initially, 2.5 ml 0.1 M phosphate buffer pH 7.6; after deoxygenation, at point D, 0.02 ml 9.5 mM dithionite added. B. As A, but with a short length of polythene tubing (1.6 mm diameter) inserted in nitrogen supply line.

PROPERTIES AND USE OF DITHIONITE

Spectrum and molar absorbance

It has long been known that dithionite absorbs ultraviolet light strongly (see *e.g.* ref. 5), but I have been unable to find a proper spectrum curve in the literature*. In fact it has a very useful band at 314 nm (see Fig. 5), the height of which is proportional to the concentration. The band disappears completely on aeration, leaving a spectrum corresponding to that of $NaHSO_3$. Measurements of absorbance at 314 nm therefore provide a convenient means of (a) testing for freedom of the system from O_2, (b) estimation of dithionite, (c) following oxidation–reduction reactions involving dithionite, (d) quantitative titration of acceptors with dithionite.

Fig. 4A, shows a test for absence of O_2. The lack of any oxidation of the dithionite, shown by the constancy of absorbance at 314 nm, demonstrates that there are no leaks and that the stream of N_2 passing through contains no O_2. A gradual fall in absorbance usually indicates that the deoxygenator needs refilling.

Fig. 4B shows a duplicate experiment, but with a short length of polythene tubing inserted in the N_2 supply line at the point P in Fig. 1. Small amounts of O_2 diffusing in through the polythene walls are carried by the N_2 stream into the cell and oxidize the dithionite. The oxidation stops when the N_2 is turned off, but when it is turned on again the O_2 that has meanwhile accumulated in the tube is swept into the cell, bringing the trace down to the line of the original curve.

* HELLSTRÖM[6] in 1937 measured absorbances at six wavelengths and drew a rough curve showing a band resembling that in Fig. 5, but less sharp and intense. Few details were given, but the gas phase was 100% CO_2.

For the spectrophotometric estimation of dithionite one must know its molar absorbance (A_M) at 314 nm, and because much depends upon the value of this quantity its determination will be described in detail.

It is not practicable to make up a solution of known concentration by weighing out the solid, which usually gives appreciably less than the theoretical concentration. The A_M was therefore determined by spectrophotometric titration against ferricyanide. This may be done in two ways. In Method (*a*) the absorbance of a stock dithionite

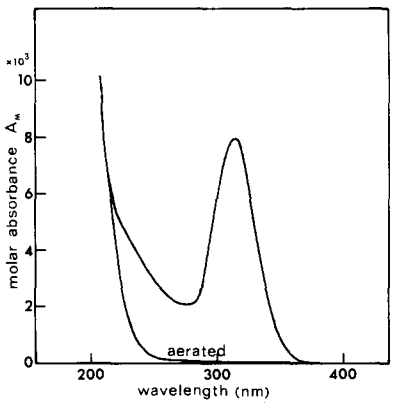

Fig. 5. Absorption spectrum of dithionite in phosphate buffer (pH 7.6).

solution at 314 nm is determined by adding small volumes to buffer solution, and (in a separate experiment) the concentration of the same dithionite solution is determined by adding small volumes to excess of ferricyanide solution, measuring the amount of ferricyanide reduced by the change of absorbance at 420 nm. In Method (*b*) small volumes of ferricyanide solution of known concentration are added to excess of dithionite solution, and the amount of dithionite oxidized is measured by the fall of absorbance at 314 nm.

Method (*a*), although it must be carried out in two parts, is theoretically the simpler, for at 420 nm there is no absorption by dithionite, by its oxidation product, or by ferrocyanide, so that the absorption is due to the ferricyanide alone. Its molar absorbance at 420 nm is known to be 1020, probably to an accuracy of about 1 % (ref. 7). No dissolved O_2 is introduced in the dithionite added. A small correction for the change in total volume on adding the dithionite may be made, but this is usually less than the experimental error.

Method (*b*) is more direct, but requires two or three small corrections. At 314 nm both ferricyanide and ferrocyanide absorb. The ferricyanide absorbance is irrelevant, since the whole of the ferricyanide added is reduced by the excess of dithionite, but the observed absorbance is then due partly to the remaining dithionite and partly to the ferrocyanide formed (A_M = 340 at 314 nm). The ferrocyanide concentration is known from the amount of ferricyanide added, and its absorbance is calculated and subtracted to give the dithionite absorbance. A small amount of the dithionite oxidation is due to traces of dissolved oxygen in the air-saturated ferricyanide added, and this should be allowed for. In calculating the results it must be borne in mind that 1 molecule of dithionite reduces 2 of ferricyanide. The rate of reduction of ferricyanide by the sulphite which is formed is negligible.

Biochim. Biophys. Acta, 226 (1971) 241–258

TABLE I

CALCULATION OF MOLAR ABSORBANCE OF DITHIONITE AT 314 nm FROM FIGS. 6 AND 7

Fig. 6A. The μmoles of dithionite present are obtained by multiplying the volume of solution added by the mean millimolar concentration calculated from Fig. 6B. The resulting millimolar dithionite concentration is given on dividing by the volume, and the A_M of dithionite is obtained by dividing the absorbance by this concentration and multiplying by 1000.

Point	*Total vol. (ml)*	*Absorbance*	*Dithionite present (μmoles)*	*Dithionite concn. (mM)*	A_M
e	2.50	0	0	0	
f	2.51	0.264	0.0825	0.0329	8020
g	2.52	0.529	0.165	0.0655	8070

Fig. 6B. The millimolar concentrations of ferricyanide present at each point are given by dividing the absorbance by 1.020, the millimolar absorbance of ferricyanide at 420 nm. Multiplication by the volumes gives the μmoles present. Δ denotes the μmoles of ferricyanide reduced by each addition, and the equivalent in μmoles of dithionite is half this. Division by the volume added gives the millimolar concentration of the dithionite solution. The first step was too small to give accurate results, and it was excluded from the calculation of the mean.

Point	*Total vol. (ml)*	*Absorbance*	*Ferricyanide concn. (mM)*	*Ferricyanide present (μmoles)*	*Δ Ferricyanide (μmoles)*	*Dithionite (μmoles)*	*Calc. concn. of stock dithionite (mM)*	*Mean (mM)*
a	2.50	0.743	0.728	1.82				
					0.15	0.075	(7.5)	
b	2.51	0.680	0.670	1.67				
					0.48	0.24	8.0	
c	2.54	0.476	0.467	1.19				8.25
					0.51	0.255	8.5	
d	2.57	0.272	0.267	0.68				

TABLE I (continued)

Fig. 7. The observed absorbance is due partly to dithionite and partly to the ferrocyanide formed. The latter is equal to the known amount of ferricyanide added, and its absorbance is equal to its millimolar concentration multiplied by its millimolar absorbance (0.340). On subtracting this from the observed absorbance, the absorbance due to dithionite is obtained. The amount of dissolved O_2 added with the air-saturated ferricyanide solution is obtained from the solubility at room temperature (about 0.3 μmole/ml). The total amount of dithionite oxidized is the sum of that oxidized by ferricyanide and by O_2, and the change of absorbance at each step divided by the change of concentration gives the A_M.

Point	*Total vol. (ml)*	*Absorbance (A)*	*Ferrocyanide concn. (mM)*	*A due to ferrocyanide*	*A due to dithionite*	*ΔA due to dithionite*	*Ferricyanide added (μmoles)*	*Dithionite (μmoles)*	*O_2 added (μmoles)*	*Dithionite oxidized (μmoles)*	*Change of of dithionite concn. (mM)*	*A_M*
a	2.53	0.684	0	0	0.684							
						0.153	0.091	0.045	0.003	0.048	0.0189	8095
b	2.54	0.544	0.0370	0.0126	0.531							
						0.297	0.182	0.091	0.006	0.097	0.0379	7840
c	2.56	0.272	0.111	0.038	0.234							

Table I shows calculations of A_M from experiments by Method (*a*) (Fig. 6) and Method (*b*) (Fig. 7), done with the same dithionite solution. The ferricyanide solution was made up from crystals of $K_3Fe(CN)_6$ (A.R. grade) from which the surface layer had been scraped off in order to remove any decomposition products.

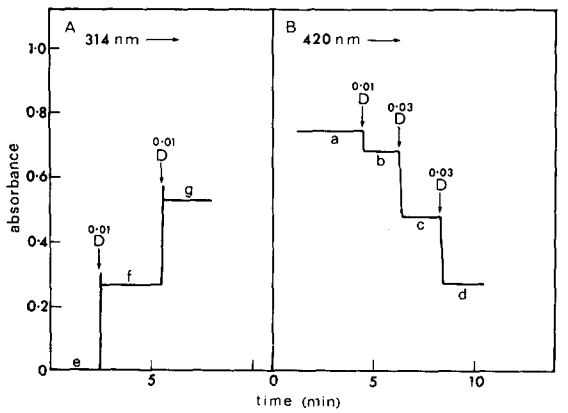

Fig. 6. Determination of molar absorbance of dithionite at 314 nm by oxidation with ferricyanide (Method (*a*)). A. Initially, 2.5 ml 0.1 M phosphate buffer (pH 7.6). At points D, addition of 0.01 ml of dithionite solution of concentration determined in B as 8.25 mM. B. Initially, 2.3 ml phosphate buffer (pH 7.6) + 0.2 ml 10 mM ferricyanide. At points D, additions of the dithionite solution used in A.

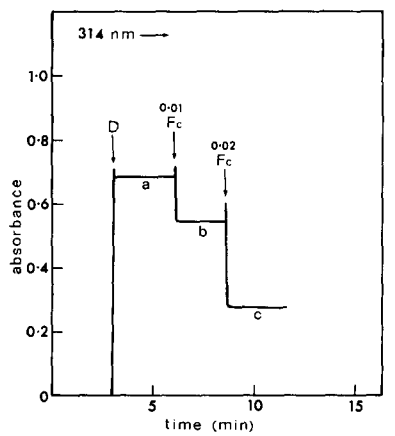

Fig. 7. Determination of molar absorbance of dithionite by oxidation with ferricyanide (Method (*b*)). Initially: 2.5 ml 0.1 M phosphate buffer (pH 7.6). At point D, 0.03 ml dithionite (about 7 mM) added. At points Fc 0.01 ml and 0.02 ml 9.1 mM ferricyanide added.

The mean of the 4 values of A_M is 8000 within experimental error. This is in agreement within a few per cent with results obtained with a variety of other acceptors (see Fig. 9 of the following paper[8]), but the accepted molar absorbances of some of these acceptors are probably less accurately known than that of ferricyanide, and the results with ferricyanide are considered to be more reliable.

Knowledge of the A_M of dithionite gives a convenient method for the titration of

acceptors even without the necessity for a standardized dithionite solution. All that is needed is, after removal of O_2 from a measured volume of buffer, to inject enough dithionite solution to give a convenient absorbance at 314 nm. The change of absorbance on adding a small amount of acceptor then gives the amount of dithionite oxidized by the acceptor. This assumes that the reduced acceptor does not absorb at 314 nm; if it does, a correction must be made as for ferrocyanide in Table I.

Reaction of dithionite with acceptors

For comparison with the behaviour of reduced flavins towards acceptors, to be described in the following paper, it is helpful to know how the same acceptors react with a fairly general chemical reducer, such as dithionite.

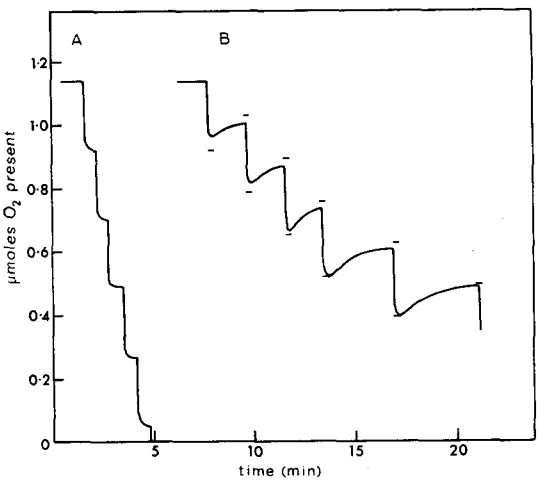

Fig. 8. Reaction of dithionite with excess of O_2, measured with O_2 electrode. A and B. Initially, 4 ml 0.1 M phosphate buffer (pH 7.6), air-saturated. At each step 0.01 ml of 21.5 mM dithionite was added. B is a repetition of A, but with a small amount of catalase added initially.

Oxygen

It has long been known that dithionite reacts very rapidly with O_2, and the velocity is too high to be measured by the methods used here. Fig. 8, which shows an experiment done, not with the spectrophotometer but with the O_2 electrode, gives at A the effect of a series of successive additions of 0.01-ml portions of a dithionite solution to 4 ml of buffer, initially saturated with air and containing 1.14 μmoles of dissolved O_2. The concentration of the dithionite solution was 21.5 mM, so that each 0.01-ml portion contained 0.215 μmoles, and it can be seen that each molecule of dithionite reduces one molecule of O_2.

If the O_2 were reduced to water, two molecules of dithionite per O_2 molecule would have been required. The explanation is given by curve B, which is a duplicate of A, but with the initial addition of a small amount of catalase. At each step the lower mark represents 1 molecule of O_2 per molecule of dithionite, measured from the end-point of the previous step, and the upper mark represents half of this. It is evident that 1 molecule of O_2 is first taken up, as in A, and that in the presence of catalase half this quantity is then released by the decomposition of H_2O_2. Thus in the absence of catalase the reaction is

$$Na_2S_2O_4 + O_2 + 2\,H_2O \longrightarrow 2\,NaHSO_3 + H_2O_2 \quad (1)$$

with a quantitative reduction of O_2 to H_2O_2. In B this is followed by the catalase reaction

$$2\,H_2O_2 = 2\,H_2O + O_2 \quad (2)$$

so that the overall reaction is

$$2\,Na_2S_2O_4 + O_2 + 2\,H_2O = 4\,NaHSO_3. \quad (3)$$

KEILIN AND HARTREE[9] detected the formation of some H_2O_2 during the oxidation of dithionite by O_2, but I am not aware that it has been suggested that the reaction is quantitative. Indeed in two fairly recent papers[10,11] on the kinetics of the process there is no mention of peroxide and the reaction is written otherwise. The formation of peroxide must be borne in mind in interpreting experiments on biological oxidation systems where dithionite is used as a reducer and the excess is removed by aeration.

It will be noticed that the first steps of Curve B fall a little short of the theoretical end-points. This is probably due to the oxidation by H_2O_2 and catalase of a trace of alcohol present in the catalase preparation, but this is soon used up and the later steps give theoretical results. KEILIN AND HARTREE[12] found that most catalase preparations contain persistent traces of alcohol.

In Fig. 8A O_2 is present in excess, so that at the end of each step there is no dithionite left and the peroxide remains. Fig. 9 shows the opposite conditions. Here small amounts of O_2 dissolved in water are added to excess of dithionite, and the oxidation of dithionite is followed by the fall in absorbance at 314 nm. There is a very rapid oxidation of 1 molecule of dithionite per molecule of O_2 in accordance with Eqn. 1, but here the peroxide oxidizes a second molecule of dithionite in a much slower phase, clearly distinguishable from the first phase, so that twice as much dithionite is oxidized under these conditions. Fig. 10 shows directly the oxidation of dithionite by H_2O_2 added as such. The rate of the reaction is of the same order as the second phase in Fig. 9, and the line on the right shows the theoretical amount corresponding to the

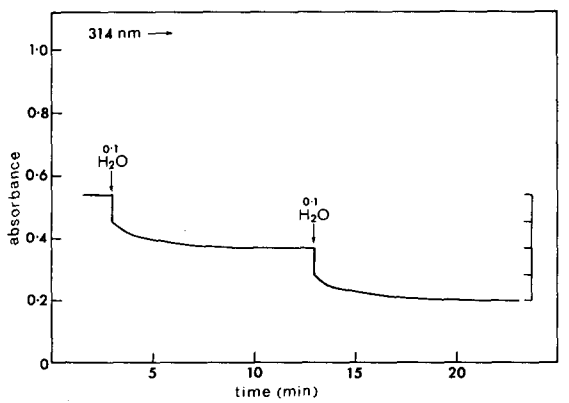

Fig. 9. Reaction of O_2 with excess of dithionite. Initially, 2.5 ml 0.1 M phosphate buffer (pH 7.6) + 0.02 ml 8.5 mM dithionite. At arrows, 0.1 ml air-saturated water (containing 0.027 μmole O_2) added. Each division of the scale on the right corresponds to the oxidation of 1 molecule of dithionite per molecule of O_2 added.

amount of peroxide added. It is interesting that dithionite is oxidized by O_2 so much more rapidly than by H_2O_2.

In reducing O_2 to H_2O_2, dithionite resembles the reduced forms of flavins and dyes, as well as several other reducing systems.

Other acceptors

Dithionite reduces the majority of the better known acceptors very rapidly, in fact too rapidly to be measured by the present method. The aim of this work was not to determine the rate constants of a large number of reactions, but to get a general idea of the specificity of the non-enzymatic reduction of acceptors by dithionite and reduced flavins, for comparison with reduced flavoproteins. It is not necessary to show curves for all cases in which the reaction is rapid and straightforward, but in order to

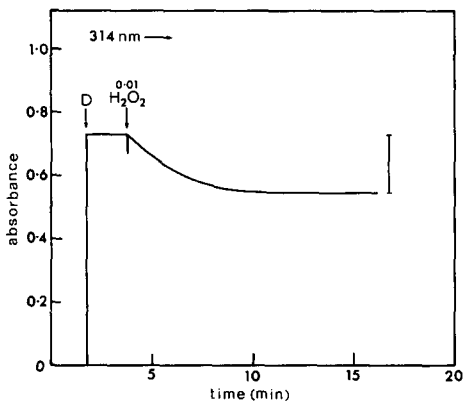

Fig. 10. Oxidation of dithionite by H_2O_2. Initially, 2.5 ml 0.1 M phosphate buffer (pH 7.6). At first arrow 0.02 ml 12 mM dithionite, and at second arrow 0.01 ml 5 mM H_2O_2, added. The line on the right gives the calculated absorbance change, assuming that 1 molecule of H_2O_2 oxidizes 1 molecule of dithionite.

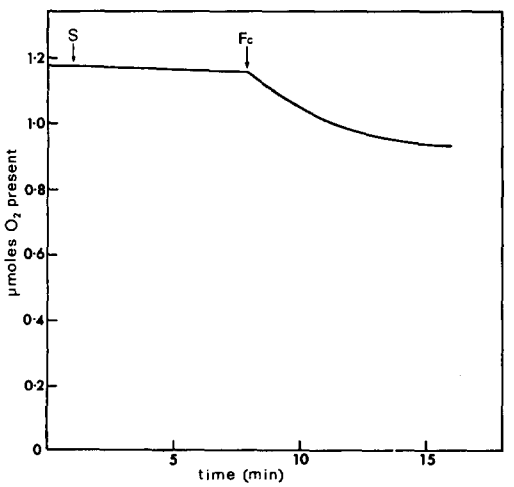

Fig. 11. Catalysis by ferricyanide of oxidation of sulphite by O_2, measured with O_2 electrode. Initially, 4.3 ml 0.1 M phosphate buffer (pH 7.6). At point S, 0.03 ml 50 mM Na_2SO_3, and at Fc 0.1 ml 10 mM ferricyanide, added.

facilitate comparison of dithionite with reduced flavins the results are included in Fig. 9 of the following paper[8].

Five types of behaviour of acceptors towards dithionite can be distinguished.

Type 1 (O_2, FMN, FAD, methylene blue). Very rapid reduction of 1 molecule of acceptor by 1 molecule of dithionite, due to the fact that these acceptors require two equivalents for their reduction and do not react detectably with the sulphite formed from the dithionite. O_2 scarcely reacts with sulphite at all under the present conditions, although the reaction is catalysed by ferricyanide (Fig. 11); the reaction between H_2O_2 and sulphite is also too slow to affect the stoichiometry. The reduction of many flavoproteins by dithionite takes place by two successive 1-equivalent steps, with the intermediate formation of a semiquinone, but with free flavins the amount formed is too small to be shown by the spectra under these conditions (Fig. 4 of the next paper[8]). No reduction of free flavins by sulphite could be seen (Fig. 12), and al

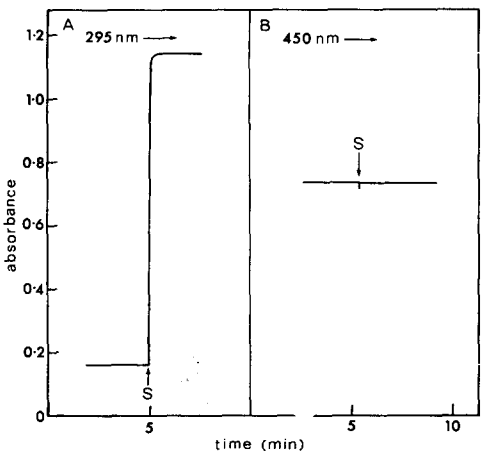

Fig. 12. Reaction of sulphite with FMN and benzoquinone. Part A: initially, 2.0 ml 0.1 M phosphate buffer pH 7.6 + 0.5 ml 0.35 mM FMN. Part B: initially, 2.0 ml 0.1 M phosphate buffer pH 7.6 + 0.5 ml 2 mM benzoquinone. At points S, 0.01 ml 100 mM Na_2SO_3 added.

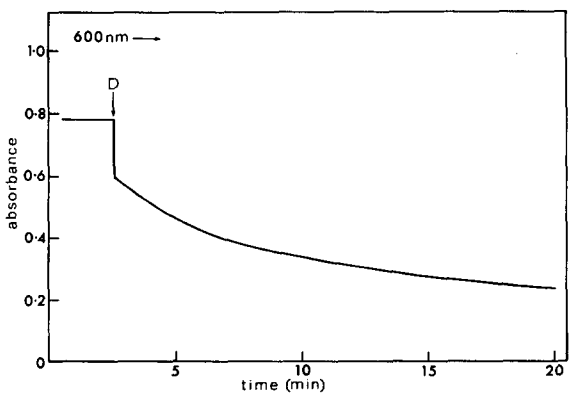

Fig. 13. Reduction of DCIP by dithionite. Initially, 2.5 ml 0.1 M phosphate buffer pH 7.6 + 0.03 ml 3.2 mM DCIP. At point D, 0.031 ml 0.693 mM dithionite added. The dithionite was originally 1.30 mM, but had become partially oxidized during storage.

though flavins form complexes with sulphite[13] these are so highly dissociated (equilibrium constant about 2 M) that their amount is negligible in the present experiments. The reduction of methylene blue has the appearance of taking place in two steps, since the rapid 2-equivalent reduction is followed by a second phase giving a slower increase of absorbance (Fig. 5 of the next paper[8]). This, however, is an artifact; reduced methylene blue is insoluble, and the second phase is due to its crystallizing out as a fine cloud of minute needles, which scatters some of the light.

Type 2 (ferricyanide, cytochrome *c*). Very rapid reduction, but of 2 molecules of acceptor by 1 of dithionite, since these acceptors require only 1 reducing equivalent.

Type 3 (benzoquinone, dichlorophenolindophenol (DCIP), phenazine methosulphate (PMS). Very rapid reduction of 1 molecule of acceptor by 1 molecule of dithionite, followed by a slower reduction of a further molecule of acceptor by the molecule of sulphite formed. In the case of benzoquinone the reaction with sulphite is so rapid that the two steps cannot be distinguished by this method (Fig. 12), and the reaction appears to be a direct reduction of 2 molecules of quinone (4 equivalents) by 1 of dithionite. With the other two acceptors the sulphite reaction is much slower and the two steps are clearly resolved. Fig. 13, with DCIP, however, illustrates a stoichiometric error which may be encountered with acceptors of this type. In this experiment a solution of dithionite was used that had become partly oxidized; originally it was 1.30 mM with respect to dithionite, but when it was used it was 0.693 mM. At the point D therefore 0.0215 μmoles of dithionite were added, and the drop in absorbance at 600 nm shows that 0.0217 μmoles of DCIP were reduced in the rapid step, taking the A_M of DCIP at the accepted value of 21000. In the slow phase that followed, 0.048 additional μmole of DCIP were reduced at the estimated end-point. If the previous history of the dithionite solution were unknown, it would be concluded that 1 molecule of dithionite had reduced 3 molecules of DCIP overall. But in fact, in addition to the 0.0215 μmole of sulphite formed in the first step, 0.019 μmole more were added with the dithionite, this being the amount of sulphite formed in the original oxidation of 0.031 ml of the dithionite solution from 1.30 to 0.693 mM. There was also a small amount of sulphite in the solid preparation of dithionite, so that the the total amount of sulphite would have been about 0.045 μmole, accounting approximately for the 0.048 μmole of DCIP reduced in the slow phase. In fact therefore 1 molecule of dithionite reduces 2 of DCIP in the overall reaction, not 3. Because of this source of error, acceptors of Type 3 are not very suitable for stoichiometric work with dithionite.

Type 4 (H_2O_2, NAD^+). Slow reduction, measured in minutes rather than seconds or milliseconds, of 1 molecule of acceptor by 1 of dithionite.

Type 5 (nitrate). No reduction by dithionite, although readily reduced by certain flavoproteins.

In the following paper[8] (Part II) the behaviour of free reduced flavins towards these acceptors is compared with that of dithionite.

REFERENCES

1 M. DIXON AND P. KENWORTHY, *Biochim. Biophys. Acta*, 146 (1967) 54.
2 G. P. FOUST, B. D. BURLEIGH, Jr., S. G. MAYHEW, C. H. WILLIAMS, Jr., AND V. MASSEY, *Anal. Biochem.*, 27 (1969) 530.
3 B. D. BURLEIGH, Jr., G. P. FOUST AND C. H. WILLIAMS, Jr., *Anal. Biochem.*, 27 (1969) 536.

4 L. F. FIESER, *J. Am. Chem. Soc.*, 46 (1924) 2639.
5 M. DIXON, R. HILL AND D. KEILIN, *Proc. Roy. Soc. London, Ser. B*, 109 (1931) 29.
6 H. HELLSTRÖM, *Z. Physiol. Chem.*, 246 (1937) 155.
7 C. H. WILLIAMS AND H. KAMIN, *J. Biol. Chem.*, 237 (1962) 587.
8 M. DIXON, *Biochim. Biophys. Acta*, 226 (1971) 259.
9 D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc. London, Ser. B*, 124 (1938) 397.
10 R. G. RINKER, T. P. GORDON, D. M. MASON, R. R. SAKAIDA AND W. H. CORCORAN, *J. Phys. Chem.*, 64 (1960) 573.
11 J. A. MORELLO, M. R. CRAW, H. P. CONSTANTINE AND R. E. FORSTER, *J. Appl. Physiol.*, 19 (1964) 522.
12 D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc. London, Ser. B*, 119 (1936) 141.
13 F. MÜLLER AND V. MASSEY, *J. Biol. Chem.*, 244 (1969) 4007.