associated with the mitochondria and is most probably associated with the microsomes.

2. The possibility of the presence of the relaxing factor in a third particle of intermediate density cannot be excluded by these studies.

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The Oxidation of Reduced Flavin Mononucleotide by Molecular Oxygen

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Reduced flavin mononucleotide is active as a 'luciferin' in the bacterial bioluminescent system (Hastings & McElroy, 1955; McElroy & Green, 1955).

The components required for light-production are luciferin, luciferase, a long-chain aliphatic aldehyde and oxygen. The work described in this paper was undertaken as a preliminary to a study of the kinetics of the bacterial luminescent system.

The reaction of reduced flavin mononucleotide with oxygen has been studied by Gutfreund (1960) who interpreted his kinetic findings in terms of the formation of a flavin-oxygen complex whose breakdown was rate-limiting. He found no spectroscopic evidence for the transient existence of intermediate species, either the reduced flavin-oxygen complex or the species observed by Beinert (1956), in equilibrium mixtures of both oxidized and reduced

riboflavin and oxidized and reduced flavin mononucleotide.

Although our kinetic observations on the reaction with oxygen generally agree well with those of Gutfreund (1960), we have been able to observe intermediate compounds similar to those seen by Beinert (1956), and have also found that the reaction is autocatalytic. Some additional observations on the part played by hydrogen peroxide and on the coupling of flavin oxidation to luminol chemiluminescence are presented.

EXPERIMENTAL

Materials

Flavin mononucleotide (FMN) was obtained from Sigma Chemical Co., and 5-amino-2,3-dihydro-1,4-phthalazine-dione (luminol) from Eastman Organic Chemicals, Rochester 3, N.Y., U.S.A. Other reagents were of analytical

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quality. Hydrogen and nitrogen were obtained from the Matheson Co., Joliet, Ill., U.S.A. These were of pre-purified grade and contained less than 10 p.p.m. of oxygen. They were used directly from the cylinders without attempts at further purification. Catalase was obtained from Worthington Mfg. Co., Freehold, N.J., U.S.A.

Methods

Rapid spectrophotometric observations were made with a stopped-flow apparatus similar to that used by Gibson & Antonini (1960). This allowed path-lengths of up to 2 cm. to be used with continuous spectrophotometric recording of reactions from 3 msec. after mixing onward. The apparatus differed in detail in the substitution of a Bausch and Lomb grating monochromator (HD 9328) for the Hilger D248 prism instrument, and in the use of a Tektronix oscilloscope type 532 in place of the Cossor 1049. The oscilloscope traces were photographed with a Du Mont oscilloscope camera type 2620 on Polaroid projection film type 46-L; it was convenient to use a second oscilloscope with a long-persistence screen to allow visual observation of the oscilloscope traces during photography. An RCA type 7102 photomultiplier was used for observations in the near-infrared region of the spectrum.

Observations of luminescence were made with the stopped-flow apparatus by substituting a special observation chamber for the 2 cm. capillary described by Gibson & Antonini (1960). This luminescence chamber consisted of a straight-through 2 mm. hole drilled in a piece of stainless steel. Opposite the photomultiplier the drilling was milled out to give an approximately cubical chamber of 4 mm. side; one face of the cube was made up by cementing a plane glass observation window in position with epoxy cement (Araldite; Aero Research Ltd., Duxford, Cambridge). In this way a fair volume of solution could be observed in a reasonably thin layer.

Static spectrophotometric observations were made with a Zeiss PMQ II or with a Cary model 14 recording spectrophotometer.

Preparation of reduced flavin mononucleotide solutions. Several methods were used, none of which was entirely satisfactory.

(1) Reduction by hydrogen with platinum as catalyst. Rapid reduction may be obtained by using platinized asbestos, but re-oxidation of flavin is difficult to avoid in the manipulations required to remove the asbestos after reduction (filtering or centrifuging). Reduction is more slowly achieved with platinum wire as catalyst. With 20 ft. of platinum wire (0.001 in. diam.) in 40 ml. of 0.1 mM-FMN dissolved in water, about 1½ hr. must be allowed to obtain about 90% reduction. The greatest drawback of the method is the loss of flavin in irreversible changes, which, as noted by Lowe & Clark (1956), increases progressively with time. This effect is most readily observed by following the decline in *E* at 450 mμ after re-oxidation of FMNH₂. The loss may be associated with changes after re-oxidation of flavin rather than with further reduction of FMNH₂, since much greater and more rapid losses may be obtained by bubbling hydrogen contaminated with a small amount (1%) of oxygen through a FMN solution in the presence of platinum. In these circumstances the flavin remains in the oxidized form but there is a rapid fall in *E* at 450 mμ.

(2) Reduction with dithionite. This method is quick and convenient, and was used by Gutfreund (1960), who added

excess of dithionite and back-titrated with oxygenated water. We have preferred to titrate forwards, putting the flavin solutions in a glass tonometer with stopcocks at both ends. This is repeatedly evacuated and filled with pure nitrogen or hydrogen, and a syringe (2 ml.), filled with dilute (about 1 mg./ml.) dithionite solution and fitted with a long (6 in.) needle, is passed through one of the stopcocks and is used to titrate the flavin. The progress of the titration may be followed by observing the fluorescence quenching that occurs upon reduction. In this there are, however, two difficulties. If all dithionite is removed (as in Gutfreund's procedure) and some small amount of oxidized flavin is therefore present after back-titration, the FMN, as will be shown later, acts as a catalyst for, and also alters the time course of, the reaction of reduced flavin with oxygen. If an excess of dithionite is present the concentration of oxygen available for reaction with flavin becomes uncertain. Further, dithionite (or some oxidation product) is an effective catalyst of the reaction (see Fig. 10).

Buffers. All experiments (unless otherwise specified) were carried out in a buffer prepared by mixing 2M-K₂HPO₄ with 2M-NaH₂PO₄ in the proportions 5.5:10, and diluting 1 part of the mixture with 19 parts of water. This solution is described as phosphate buffer, pH 6.3.

RESULTS

Course of the reaction of reduced flavin mononucleotide with oxygen as followed by changes in E 450 mμ. A typical example of a reaction record obtained by the stopped-flow method is given in Fig. 1 (A). Inspection of this curve suggests that

Fig. 1. (A) Reaction of 0.025 mM-FMN₂ with 0.125 mM-oxygen in the stopped-flow apparatus; 2 cm. path, 450 mμ, 0.1M-phosphate buffer, pH 6.3. (B) Conditions and reactants were as for (A), except that the oxygen was dissolved in 0.025 mM-FMN solution instead of in water.

the reaction speeds up as it proceeds, and this impression is borne out when the value for the apparent first-order rate constant of the decline in E is calculated over successive time-intervals and plotted against time (Fig. 2). When a reaction speeds up in this way, an obvious possibility is that a product is acting as an accelerator. With FMNH₂, the expected products are FMN and hydrogen peroxide. The effect of adding FMN to the reaction mixture is shown in (B) of Figs 1 and 2, which indicate that FMN does speed the oxidation of FMNH₂. As FMN is stable in the presence of oxygen, it seems likely that it does so by reacting with FMNH₂ to give a compound able to react with oxygen faster than FMNH₂ itself.

Effect of flavin concentration. Experiments on the effect of varying the concentration of reduced flavin showed that within the range 0.01–0.2 mM the reaction is somewhat slower (decreased by about 10%) at the lowest concentrations and the increase in rate constant as the reaction proceeds is less marked. The differences observed are illustrated in Fig. 12. There is a more marked increase at still higher concentrations, but the reaction can no longer be followed at 450 m μ because of the high extinction. At the longer wavelengths that must be used, interference from compounds other than FMN and FMNH₂ cannot be safely neglected.

Variation in wavelength. The reaction of the same oxygen–FMNH₂ mixture was followed with six different wavelengths from 375 to 500 m μ . The total changes correlated well with those found by static differential spectrophotometry (Fig. 3); with the possible exception of observations at 500 m μ , the time-course of the reaction was identical within experimental error at all six wavelengths.

Effect of varying oxygen concentration. If a dilute (0.04 mM) flavin solution is mixed with progressively smaller amounts of oxygen and the reciprocal of the first-order rate constant at, say, 50% oxidation is plotted against the reciprocal of the oxygen concentration, a straight line is found, which, on extrapolation, intersects the ordinate yielding an apparent maximum rate of oxidation of FMNH₂ at infinite oxygen concentration. This result, which confirms the finding of Gutfreund (1960), is illustrated in Fig. 4. The numerical value is in excellent agreement with his figure, though too much significance should not be attached to this, since the value depends on the point in the reaction chosen for determining the rate constant, and on the concentration of flavin used. In addition, the shape of the reaction record is dependent on oxygen concentration; the autocatalytic effect is least with the highest concentrations (see Fig. 11).

When a relatively high concentration of reduced flavin is mixed with various amounts of oxygen in

the stopped-flow apparatus, two distinct results may be obtained. If the oxygen is in excess (more than 1 mole of oxygen/mole of flavin) a straightforward reaction of the type already described occurs. When the amount of oxygen used falls

Fig. 2. Changes in the apparent first-order rate constant calculated over successive intervals of 25 msec. for the data of Fig. 1.

Fig. 3. Difference spectrum FMN–FMNH₂ obtained: (1) by reducing 0.027 mM-FMN with hydrogen and platinum wire as catalyst in a 1 cm. spectrophotometer cell and obtaining the difference spectrum from that of oxidized flavin directly with a recording spectrophotometer (pH 6.3, 25°; these results are plotted as a continuous line); (2) by allowing FMNH₂ prepared as described above to react with oxygen (0.125 mM) in the stopped-flow apparatus and following the reaction with light of various wavelengths (these results are plotted as ○).

below 1 mole/mole a biphasic reaction takes place, with an initial rapid change followed by a slow phase.

At 3° the second phase is extended sufficiently so that a reasonable separation from the first phase may be obtained. In these circumstances the rapid phase corresponds to the oxidation of 1 mole of flavin/mole of oxygen added. It seemed likely that the slow phase was due to reaction with hydrogen peroxide formed in the rapid phase of the reaction. This has been verified by adding catalase to the reaction mixture. The difference in rate between the slow and rapid phases is much lessened (Fig. 5), but not abolished, by the addition of catalase. As a further check, the rate of reaction between hydrogen peroxide and FMNH₂ has been measured directly and was about one-hundredth of that of the reaction with oxygen. It, too, is autocatalytic.

Spectroscopic changes in the long-wavelength region during flavin oxidation. Beinert (1956) measured the spectra of equilibrium mixtures of FMN and FMNH₂ and demonstrated absorption in the region between 550 and 1200 mμ. This absorption and its alteration with temperature and concentration were explained by supposing that a compound is formed by interaction between FMN and FMNH₂ that absorbs in the region centred at about 570 mμ, and that the dimer of this compound possesses an absorption maximum at about 900 mμ.

Fig. 4. Reciprocal of the first-order rate constant for the reaction at 25° of 0.02 mM-FMNH₂ with oxygen at various concentrations plotted against the reciprocal of the oxygen concentration. The buffer was 0.1 M-phosphate, pH 6.3.

By using suitable concentrations of flavin and of oxygen, transient spectral changes in the long-wavelength region have been found which correlate closely with Beinert's observations, showing that the equilibria between FMN, FMNH₂, the 570 mμ-absorbing species and its dimer are approximated to in periods of the order of 0.1–1 sec. (i.e. less than the time required for flavin oxidation). Attempts were made to measure the rate of interaction of FMN and FMNH₂ directly by mixing oxygen-free solutions of these compounds in the stopped-flow apparatus. The reaction between them to give the compounds absorbing at 570 and 900 mμ was always complete at the earliest time of observation (3 msec.) permitted by the stopped-flow apparatus.

These observations closely parallel those of Burn & O'Brien (1959), who made a detailed study of the spectroscopic changes during reduction of riboflavin by dithionite, and found the transient appearance of intermediate compounds in that reaction also.

An attempt has been made to correlate the change seen at long wavelengths with the progress

Fig. 5. Graphical representation of the changes in E at 505 mμ after mixing 0.2 mM-FMNH₂ with 0.14 mM-oxygen in the stopped-flow apparatus at 20°, 0.1 M-phosphate buffer, pH 6.3. (A) No catalase; (B) with 1500 units of catalase/ml. (as standardized by the Worthington Mfg. Co.).

of the reaction as measured in the blue region of the spectrum. This is technically difficult because solutions strong enough to give good changes of E at 570 $m\mu$ are too dense at 450 $m\mu$, and it has been necessary to use the absorption minimum at 400 $m\mu$ instead. The range of concentrations that can be examined is further limited in the oxidation reaction by the solubility of oxygen in water. The results of one experiment are given in Fig. 6, which shows the course of E at 570 $m\mu$ during the reaction between FMNH₂ and oxygen as observed, and as calculated from the progress curve at 400 $m\mu$ on the assumption that the species absorbing at 570 $m\mu$ is in equilibrium with FMN and FMNH₂, and that its concentration is proportional to the product of their concentrations. The agreement is as good as can be expected in view of the small total change in E at 570 $m\mu$ (0.030), and the approximate basis for the calculated values.

Fig. 6. Comparison between the changes observed at 570 $m\mu$ (O) during the reaction of 0.2 mm-FMNH₂ with 0.6 mm-oxygen, 0.1 M-phosphate buffer, pH 6.3, at 25°, 2 cm. light-path, with the changes calculated (●) as described in the text from the progress curve for the reaction as measured at 400 $m\mu$ with a 4 mm. light-path.

Fig. 7. Changes in E at 570 $m\mu$ (●) and at 900 $m\mu$ (O) during the reaction of 0.42 mm-FMNH₂ with 0.6 mm-oxygen at 25°, 0.1 M-phosphate buffer, pH 6.3, 2 cm. path. Maximum change observed at 570 $m\mu$ = 0.093, at 900 $m\mu$ = 0.067.

The relation between the changes at 570 and 900 $m\mu$ has also been examined. As shown in Fig. 7, the time-course of the changes in E differs significantly at the two wavelengths. If Beinert's (1956) suggestion that E at 900 $m\mu$ is chiefly due to a dimeric form of the species absorbing at 570 $m\mu$ is correct, then, to a first approximation, E at 900 $m\mu$ should be proportional to the square of E at 570 $m\mu$. Fig. 8 shows that the relation is closely obeyed, though the points derived from the ascending limb of the curve in Fig. 7 lie slightly below those derived from the descending limb. This effect is not due to experimental error, but becomes increasingly evident as the flavin concentration is raised (Dr V. Massey, personal communication).

The luminol reaction and flavin oxidation. It has been proposed (White, 1961; Spruit-Van Der Burg, 1950) that the mechanism of luminol chemiluminescence includes the reaction of a radical with a luminol molecule to give a luminol radical, and that the luminol radical reacts with oxygen with emission of light. On this basis Totter, De Dugros & Riveiro (1960) have used the luminol reaction to demonstrate the formation of free radicals in the xanthine-oxidase reaction. As the monomeric intermediate species absorbing at 570 $m\mu$ may be a free radical, and as Beinert (1956) was able to demonstrate paramagnetic-resonance absorption in partially reduced solutions of FMN, an attempt was made to correlate luminol chemiluminescence with flavin oxidation. It was in fact found that light is produced in mixtures of FMNH₂ with oxygen in the presence of luminol, and the time-course of the

Fig. 8. Relation between E at 900 $m\mu$ and E^2 at 570 $m\mu$. Ascending limb (●) is passing from reduced to half-oxidized flavin; descending limb (O), from half-oxidized to fully oxidized flavin. For explanation see text. Conditions and concentrations are as given for Fig. 7.

light-emission, together with the progress of flavin oxidation as obtained by spectrophotometric measurement at 450 m μ , is given in Fig. 9. The light-emission consists of a brief, bright flash, which continues during the flow period and decays when flow stops, with a half-time of about 5 msec. The light-output reaches a minimum after about 15 msec. and then increases to a maximum after about 150 msec. It decays and disappears after about 0.25 sec. Apart from the initial flash it does not seem that the rate of light-production can be correlated with any spectroscopically detectable intermediate in the flavin oxidation, since the reaction is virtually complete by about the time that maximum light-output has been reached. For example, if the 570 m μ -absorbing species were a radical able to react with luminol it would be expected that the course of the light-output would follow *E* at 570 m μ . It seems likely that the main light-production observed is due to the reaction of hydrogen peroxide with luminol. Attempts to examine this suggestion quantitatively have not succeeded. The emission spectrum of luminol luminescence overlaps the absorption spectrum of oxidized flavin heavily, and the screening effect is large with the concentrations used. Further, light-production from the luminol-hydrogen peroxide reaction is influenced by oxidized flavin in a complicated way. If luminol (1 mg./ml. in 0.02N-sodium hydroxide) containing 0.4 mM-FMN is mixed with 0.1 mM-hydrogen peroxide the initial rate of light-production is four times that when the same

mixture is prepared by using 0.1 mM-hydrogen peroxide containing 0.4 mM-FMN to mix with 1 mg. of luminol/ml. in 0.02N-sodium hydroxide. Either luminol and oxidized flavin react together or hydrogen peroxide has a destructive effect on FMN. These results are not due to chemiluminescence analogous to that reported by Strehler & Shoup (1953) to occur in mixtures of riboflavin and hydrogen peroxide, as control experiments with FMN and hydrogen peroxide did not give sufficient light to be detected under our experimental conditions. In view of these difficulties the matter was not pursued further.

Catalytic effect of dithionite on oxidation of FMNH₂. If FMN is reduced with dithionite, care being taken to avoid using excess, the FMNH₂ reacts with oxygen in much the same way as does FMNH₂ prepared by using platinum wire and hydrogen (Fig. 10, A). If an amount of dithionite that is just slightly in excess of that required to reduce the FMN is added to the solution and the experiment repeated, the rate of oxidation is increased as much as five- to six-fold (Fig. 10, B). It is difficult to give precise directions for this experiment, because the result depends on the conditions used for preparing the dithionite solution, but as a rough guide it is suggested that 0.2–0.4 ml. of an 0.1% solution of dithionite be added to 10 ml. of a 0.03 mM solution of FMNH₂.

Fig. 9. ●, Light-production (arbitrary units); ○, change in *E* at 450 m μ , 4 mm. path. Conditions: 0.2 mM-FMNH₂, 0.5 mg. of luminol/ml., 0.125 mM-oxygen, 0.01N-sodium hydroxide, 30°.

Fig. 10. Effect of small quantities of dithionite on the reaction between FMNH₂ and oxygen. 4 μ M-Flavin, 0.125 mM-oxygen, 0.1M-phosphate buffer, pH 6.3. (A) No addition; (B) addition to the flavin solution of 0.02 vol. of 0.1% solution of sodium dithionite in phosphate buffer, pH 6.0, 2 cm. path, 25°, 450 m μ .

Oxidation of flavin by ferricyanide. When potassium ferricyanide was tried, the reaction was too fast to measure at pH 6.3, even when equivalent concentrations of FMNH₂ and potassium ferricyanide were used. The reaction was more than 90% complete in 3 msec. and the rate constant must be of the order of 10⁸ M⁻¹ sec.⁻¹.

DISCUSSION

The main features of the reaction to be accounted for are: (1) the autocatalytic time-course; (2) the effect of oxygen concentration on rate; (3) the effect of flavin concentration on rate; (4) the rapid equilibrium between FMN, FMNH₂ and the 570–900-mμ-absorbing species; (5) the time-course of light-emission with luminol.

So far, no entirely satisfactory scheme has been developed, but some of the features of the reaction can be dealt with quite simply.

The autocatalytic time-course, together with the effect of addition of oxidized flavin, might be explained by a scheme in which the rate of reaction with oxygen is proportional to the product of FMN and FMNH₂ concentrations. In the simplest case, where the concentrations of all flavin compounds other than FMN and FMNH₂ are small enough to be neglected by comparison, writing a = concentration of total flavin, $[FMN] = a - x$, $[FMNH_2] = x$ and $s = [I]$, in the scheme:

where I represents an intermediate semiquinone compound, and the concentration of oxygen is assumed sufficiently large so that it may be taken as constant throughout the reaction

$$\frac{ds}{dt} = k_1x(a-x) - (k_{-1} + k_2)s = 0 \quad (1)$$

the rate of FMN formation

$$= k_2s = \frac{k_1k_2x(a-x)}{(k_{-1} + k_2)} \quad (2)$$

On integration, (2) yields

$$t = \frac{(k_{-1} + k_2)}{ak_1k_2} \ln \frac{(a-x)}{x} + c \quad (3)$$

where c is the constant of integration, i.e. if the logarithm of the ratio of FMNH₂ to FMN is plotted against time, a straight line should be obtained. Equation (3) fails in three main ways: (1) the reaction would not start if fully reduced flavin were used; (2) it does not explain why the autocatalytic course of the reaction is less marked with higher oxygen concentrations; (3) in order to get a reasonable fit to the experimental results with equation (3) it is necessary to assume that much more FMN is

present initially than can be demonstrated by spectrophotometry.

These difficulties are readily met if, in addition to the autocatalytic mechanism, it is supposed that there is a direct reaction of FMNH₂ with oxygen, as proposed by Gutfreund (1960). His scheme includes the formation of an oxygen-FMNH₂ complex which breaks down to give FMN and hydrogen peroxide. This first-order breakdown step is rate-limiting at high oxygen concentrations, and so explains the dependence of rate on oxygen concentration shown in Fig. 4. The rate does not increase indefinitely with increasing oxygen concentration, but approaches a limit determined by the breakdown step.

The complete scheme for flavin oxidation is then:

where J is an intermediate flavin-oxygen complex as suggested by Gutfreund (1960).

Again writing $x = [FMNH_2]$, $y = [FMN]$, $s_1 = [I]$, $s_2 = [J]$, a = concentration of total flavin compounds, b = [oxygen] at time zero, this scheme leads to the set of equations:

$$\frac{dx}{dt} = k_{-1}s_1 + k_{-3}s_2 - k_3x(b-y-s_2) - k_1xy \quad (7)$$

$$\frac{dy}{dt} = k_4s_2 + k_{-1}s_1 + k_2s_1(b-y-s_2) - k_1xy \quad (8)$$

$$\frac{ds_2}{dt} = k_3x(b-y-s_2) - k_{-3}s_2 - k_4s_2 \quad (9)$$

$$\frac{ds_1}{dt} = a - x - y - s_2 \quad (10)$$

These cannot be solved analytically, but graphical solutions have been obtained by using a SCD 10 analogue computer (Solartron Ltd., Farnborough, Hants.). Although the equations contain six constants, the number available for curve-fitting was reduced to two by first assigning the value 24 sec.⁻¹ at 25° and pH 6.3 to k_4 , in accordance with the result of the extrapolation procedure of Fig. 4. Next, preliminary trials showed that the observed dependence of the apparent first-order rate constant on oxygen concentration, together with the time-course of the reaction at different oxygen concentrations, required that the autocatalytic mechanism (equations 4 and 5) be substantially independent of the oxygen concentration. This can be achieved by making k_2 large, so that the formation of the intermediate I is rate-limiting. The precise value of k_2 is then unimportant. The four remaining constants,

k_1 and k_{-1} , and k_3 and k_{-3} , operate in pairs, so that their ratios are more important than their absolute values.

It has been assumed that equilibrium is reached quickly between FMNH₂, oxygen and the intermediate complex J , whereas k_1 has been made rate-limiting in equations (4) and (5), k_{-1} being set equal to zero. When k_3 is large, changes in the value of k_{-1} would have little influence on the overall rate (unless k_{-1} were made of the same order of magnitude as k_3), and it is accordingly simplest to set k_{-1} at zero throughout. In fitting the curves, then, k_1 and k_3 were varied and all other constants left unchanged.

The analogue computer gives values of the variables x , y , s_1 and s_2 at any time after initiation of the reaction; the quantity observed with the stopped-flow apparatus is the change in E with time. The results of experiments in which the reaction was followed at different wavelengths show, however, that species other than FMN and FMNH₂ do not contribute significantly to light-absorption in the range 370–450 m μ , so that the change in E in this region on addition of oxygen can be taken as a measure of the amount of FMNH₂ oxidized.

It remains necessary to assign spectrophotometric properties to the intermediate compound J , the FMNH₂-oxygen complex postulated by Gutfreund. It is assumed that the spectrum of this complex (which must be present in considerable

quantities during the reaction), is similar to FMNH₂. If it resembled FMN, varying the oxygen concentration would alter the change in E observed, whereas if it were unlike either FMN or FMNH₂, the course of the reaction would appear to change when the wavelength of the observing light was altered. Possible interference from intermediate I has been neglected as, according to the scheme, it is present only in small amounts.

With these assumptions changes in y should be proportional to change in E at 450 m μ . It proved easy to obtain satisfactory agreement between theory and observation, to account for the autocatalytic time-course, for the variation in shape of curve with oxygen concentration, for the variation in rate with oxygen concentration and for the effect of flavin concentration on the rate and the shape of curve together with the effect of added flavin. Some of these points are illustrated in Figs. 11 and 12, which are reproductions of actual computer traces. It should be pointed out that the records in Figs. 11 and 12 were all computed with a single set of constants. The agreement with observation is fully as good as can be expected.

Fig. 11. Comparison between observed and computed results for the oxidation of FMNH₂ by oxygen. Curves and axes were generated by a Solartron SCD10 computer, and the experimental points added afterwards. $\circ$, 0.021 mM-FMNH₂ reacting with 0.125 mM-oxygen, at 24°, 0.1M-phosphate buffer, pH 6.3 (initial [FMN] = 1.9 μ M); $\bullet$, conditions as for $\circ$ except initial [O₂] = 0.6 mM; $\bullet$, 0.025 mM-FMNH₂ + 0.026 mM-FMN reacting with 0.125 mM-oxygen (other conditions were as for $\circ$ and $\bullet$). Computed curves were obtained from the system of equations (7) to (10) with: $k_1 = 5.8 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$, $k_{-1} = 0$, $k_3 = 7.0 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$, $k_{-3} = 1.7 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$, $k_{-3} = 1180 \text{ sec}^{-1}$, $k_4 = 24 \text{ sec}^{-1}$. Ordinate gives ΔE with scale adjusted to give the same starting point in each case.

Fig. 12. Effect of varying flavin concentration on the reaction between FMNH₂ and oxygen. $\circ$, Reaction of 0.042 mM-FMNH₂ with 0.6 mM-oxygen; 25°, 0.1M-phosphate buffer, pH 6.3. $\bullet$, Reaction of 4.2 μ M-FMNH₂ with 0.6 mM-oxygen; other conditions were as for $\circ$. Curves were computed with constants in the proportions given in the legend to Fig. 11, but each was increased by 8% above the values given there to allow for the higher temperature.

It is more difficult to relate the kinetic scheme to a chemical mechanism, to interpret the spectroscopic changes at 570 and 900 $m\mu$, and to explain the luminol reaction.

Any scheme depends on the identification of the species absorbing at 900 and 570 $m\mu$. If these are semiquinone dimer and monomer respectively (Beinert, 1956), then an additional hypothesis is needed to explain the slow rate of reaction with oxygen [expressed formally by the small value of k_1 in (9)] through the reactions of (4) and (5).

Two possibilities are: (a) the formation of a complex by the semiquinone with oxygen, analogous to J of (6); (b) the existence of a small amount of a reactive compound in equilibrium with the species absorbing at 570 $m\mu$. The identification of the compounds absorbing at 570 and 900 $m\mu$ is, of course, not yet conclusive.

It appears to be necessary to suppose that when oxygen reacts with the semiquinone 1 electron equivalent will be transferred and some hypothetical species such as a $O_2H\cdot$ radical must be postulated that can react with another molecule of flavin. With the concentrations of flavin used the time between the formation of such a radical and its encounter with another flavin molecule would be very short and its introduction would not alter the kinetic picture, though the interpretation of numerical values of the rate constants would be affected.

Similar considerations apply to the intermediate complex J , where the oxygen was thought of by Gutfreund (1960) as possibly bridging the gap between the reactive hydrogen atoms. It is also possible that the reaction proceeds by the formation of a peroxide, which might decompose with the formation of $O_2H\cdot$ and flavin radicals. As the reaction is taking place in solution, these radicals would have a high probability of reacting together rather than escaping from the solvent cage in which they are formed. The possible reactions for intermediate J would be:

Although there is no experimental evidence favouring one scheme more than the other, inspection of a molecular model suggests that the gap between the reactive hydrogens may be too large to be bridged readily by an oxygen molecule.

It is interesting that ferricyanide can act so efficiently as an oxidant. It would be expected to react to give a radical which in turn could react with a second molecule of ferricyanide. Both these reactions are apparently very rapid. The catalytic effect of dithionite may be due to the formation of radicals in the course of its reaction with oxygen

which in turn react with FMN to give flavin radicals which can themselves react rapidly with oxygen.

It seems reasonable to explain the initial flash of light in the experiments with luminol in terms of the reaction of a flavin-free radical which reacts with luminol but is taken up rapidly by oxygen. As already discussed, the delayed light-emission is not attributed to flavin compounds.

The energies of molecular orbitals in $FMNH_2$ and leucomethylene blue have been calculated by Pullman & Pullman (1959), who consider that whereas both substances should react readily with oxygen, leucomethylene blue should do so more readily than reduced flavin. More recently Orloff & Fitts (1961) have suggested that the calculations of Pullman & Pullman (1959) overestimated its probable reactivity with oxygen and found, on recalculation, that leucomethylene blue should be similar to phenothiazine and chlorpromazine in this respect. In experiments with leucomethylene blue it, like $FMNH_2$, reacts through an intermediate. Thus calculations of the readiness of the fully reduced form of either $FMNH_2$ or of methylene blue to donate electrons to oxygen cover only a part of their auto-oxidation reactions, and it would be interesting to know the theoretical position for a radical intermediate.

The picture that has been presented of the reaction of $FMNH_2$ with oxygen is complicated, but it does seem that many of its features can be accounted for in terms of partial reactions involving the transfer of single electrons.

SUMMARY

1. The oxidation of reduced flavin mononucleotide by molecular oxygen is autocatalytic.
2. Autocatalysis is due to an interaction between oxidized and reduced flavin mononucleotides which yields a compound able to react with oxygen.
3. The compounds described by Beinert (1956) in equilibrium mixtures of oxidized and reduced flavins (with absorption at 570 and 900 $m\mu$) are formed rapidly during flavin oxidation.
4. In the presence of luminol, flavin oxidation is accompanied by light-production. There is an initial flash lasting for about 5 msec., followed by a light-emission extending over 0.2 sec. The secondary light-emission is ascribed to the formation of hydrogen peroxide in the reaction mixture.
5. A scheme representing the reaction has been developed, and compared with observation by an analogue computer.

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Effects of Insulin and Diet on the Metabolism of L(+)-Lactate and Glucose by the Perfused Rat Heart

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The rat-heart preparation of Bleehe & Fisher (1954) has been used in the present investigation to study the effects of diet and insulin on the metabolism of glucose and L(+)-lactate by the beating heart when added singly and in combination to the perfusate. This preparation permits more precise measurements of cardiac metabolism under specified conditions than is possible in the isolated heart-lung preparation perfused with blood or in the intact animal. A particular advantage of the isolated-heart preparation is that maximum rates of utilization at high substrate concentrations and the competition between substrates may be measured without interference from unknown humoral factors.

METHODS

The perfusion apparatus and experimental techniques were described by Williamson & Krebs (1961). Male rats (Wistar strain) weighing between 220 and 280 g. were used. 'Well-fed' animals had unrestricted access to water and stock laboratory diet (Short & Parkes, 1949; Bruce, 1950), containing approximately 55% of carbohydrate. Starved rats were without food for 24 hr. Other rats were maintained on a high-carbohydrate diet for a week before use, and had unrestricted access to water. The high-carbohydrate diet contained approximately 67% of carbohydrate, and was composed of a paste of 200 g. of casein, 700 g. of sucrose, 50 g. of McCollum's salt mixture no. 185

(McCollum, 1917) and 100 g. of water. The initial volume of perfusate in aerobic experiments was either 17 or 20 ml. and samples (1 ml.) were removed for analysis after 15 and 75 min. In anaerobic experiments the initial volume of perfusate was increased to 50–70 ml. This ensured that the lactic acid formed by glycolysis did not overcome the buffering capacity of the bicarbonate of the perfusion fluid.

Crystalline L(+)-lactic acid was prepared by the method of Krebs (1961) and neutralized with N-NaOH. Glucose, glycogen, lactate and the oxygen consumption were measured by the methods used by Williamson & Krebs (1961).

RESULTS

Rate of uptake of L(+)-lactate. The time-course of the uptake of L(+)-lactate by the perfused heart from a fed rat was determined by measuring the concentration of lactate in the perfusion medium after 15 min., and at subsequent 30 min. intervals up to a total time of perfusion of 105 min. During this time the concentration of lactate in the perfusate fell from 5.0 to 1.4 mM, but the rate of removal of lactate remained approximately linear, and was of the order of 260 μ moles/g. dry wt./hr. (Table 1).

Effect of starvation on the metabolism of L(+)-lactate. The mean rate of lactate uptake was measured over the period between 15 and 75 min. of perfusion, and the mean glycogen content of hearts was obtained after 15 and 75 min. of perfusion. The initial concentration of lactate in the

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