Kinetic Studies on the Reaction between Native Globin and Haem Derivatives

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It was reported by Hill & Holden (1926), and confirmed by later workers, that the reaction between haematin and globin is rapid, but no numerical results appear to have been published. The reaction between peroxidase apoprotein and haematin has, however, been examined (Theorell & Maehly, 1950). It was slow, with a half-time of the order of minutes; the results with different preparations showed variations up to seven-fold in the rate.

Rossi-Fanelli, Antonini & Caputo (1958) have obtained globin preparations which on reaction with haematin yield haemoglobin derivatives with functional properties indistinguishable from those of the natural protein (Rossi-Fanelli & Antonini, 1959; Antonini & Gibson, 1960). These authors have suggested that on recombination the haematin molecules return to just the same positions as those occupied in the natural protein. It is of some interest to know how rapidly this reaction can occur, as it bears on the accessibility of the haem-binding sites of the globin, though, of course, kinetic data alone cannot serve to locate these in relation to the remainder of the molecule.

The rate of reaction of native human globin with haematin, carboxyhaem and monocyanoide haem has been measured under several experimental conditions. Parallel experiments have also been carried out with native human-myoglobin globin. A preliminary account of some of these experiments has already been presented by Gibson, Rossi-Fanelli & Antonini (1959).

EXPERIMENTAL

Materials

Potassium cyanide was of analytical quality (British Drug Houses Ltd.), sodium dithionite was Manox brand (supplied by Hardman and Holden, Miles Platting, Manchester).

Experiments at pH 9.1 were carried out with a borate buffer prepared by dissolving 1.9 g. of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ in 100 ml. of water. Buffer of pH 10.6 was prepared according to Kolthoff (1925).

Haematin solutions were prepared by dissolving crystalline proto- or meso-haemin in 0.01 N-NaOH to give a solution of about 1 mM. This solution was further diluted as required with buffer to give the working solution.

Carboxyhaem solutions were prepared by reducing the necessary volume of stock haematin solution with a few crumbs of sodium dithionite (about 0.5 mg./ml.) and diluting with deoxygenated buffer which had been equilibrated with a suitable partial pressure (usually between 100 and 200 mm. Hg) of carbon monoxide.

Dicyanide haem solutions were prepared by dissolving the required weight of KCN in borate buffer, adding 0.5 mg. of sodium dithionite/ml., and then introducing the volume of stock haematin solution necessary to give the appropriate final concentration of dicyanide haem.

Myoglobin globin was prepared from crystalline human myoglobin by the method of Theorell & Akeson (1955).

Other materials and buffers were as described by Antonini & Gibson (1960).

Methods

Stopped-flow kinetic determinations were carried out with the apparatus of Gibson (1954). For many experiments with low concentrations of haematin and globin, a special observation tube was used which gave a light-path length of 2 cm. in place of the normal 2 mm. tube. This is constructed entirely of stainless steel except for the two acrylic resin plugs (see Fig. 1), through which the light beam enters and leaves. The observation tube itself is surrounded by a water-jacket to control the temperature and the whole assembly can be fitted into the stopped-flow apparatus interchangeably with the ordinary observation tube. The volume of fluid observed is 63 mm.³, which, with the flow rates used, corresponds to a time difference of about 3 msec. between the oldest and newest portions of mixture being studied in any single stopped-flow determination. Control experiments in which myoglobin was allowed to react with different concentrations of carbon monoxide showed that the mean delay between mixing of the reagents and their arrival in the observation tube was 3 msec.

The reactions were followed spectrophotometrically with monochromatic light from a Hilger D. 246 monochromator.

The other procedures required were carried out as described by Antonini & Gibson (1960).

Concentrations of globin are stated in terms of a molecular weight of 16 500. The particle size was in many cases a multiple of this value (Rossi-Fanelli *et al.* 1958).

RESULTS

Combination of haematin with globins

The time course of the early part of the reaction of native human globin from haemoglobin with protohaematin is shown in Fig. 2. The concentration of haematin was 33 μM , and the globin concentration varied from 16 to 132 μM . Except for

Fig. 1. Sectional diagram of observation tube for stopped-flow apparatus with 2 cm. light-path length.

Fig. 2. Reaction of protohaematin with human globin. Haematin, $33 \mu\text{M}$ after mixing. Temp. 21° ; 0.1 M -phosphate buffer, pH 7.1. Observations were made at $406 \text{ m}\mu$, light path 2.38 mm . Globin: ○ (lower curve), $17 \mu\text{equiv.}$; ○ (upper curve), $33 \mu\text{equiv.}$; ×, $66 \mu\text{equiv.}$; ●, $132 \mu\text{equiv.}$

the lowest globin concentration, where only 0.5 equiv. of globin was added, the experimental curves do not vary significantly as the globin concentration is increased. The reaction is biphasic, about 20% of the total taking place in the first 0.15 sec., whereas about 15 sec. is required for 90% completion.

Qualitatively, the biphasic time course and the

lack of effect of change in globin concentration may be explained by supposing that the results depend chiefly on the behaviour of the haematin. Shack & Clark (1947) have shown that haematin in aqueous buffer solutions is polydisperse, with particle weights ranging up to many thousands, representing aggregates of up to 100 haematin molecules. The initial rapid change of extinction may be explained as due to the combination of monomeric haematin with globin, and the slower long-continuing change as due to the dispersal of haematin aggregates followed by the combination of the dispersed haematin with other specific sites on the globin molecule. Although no definite value for the velocity constant of the haematin+globin reaction can be given, a lower limit can be set, at $5 \times 10^4 \text{ M}^{-1} \text{ sec.}^{-1}$.

Experiments similar to those shown in Fig. 2 have been carried out on the reaction of haemoglobin globin with meso- and deuterio-haematin, and in addition the reaction of myoglobin globin with proto-, meso- and deuterio-haematin has been examined. These results are summarized in Fig. 3, which shows that the rates with meso- and deuterio-haematin are of the same general order as those for haemoglobin globin combining with protohaematin. The reaction of myoglobin globin with protohaematin is not shown in Fig. 3: in an experiment in which haemoglobin globin and myoglobin globin were compared side by side the progress curves for the two proteins agreed within experimental error and would lie closely upon the curve in Fig. 3 representing the reaction of mesohaematin with haemoglobin globin.

The rate of reaction observed in different experiments, carried out under apparently similar conditions, varied up to threefold. These differences,

which are similar to those found by Theorell & Mæhly (1950) with peroxidase, may be due to variations in the degree of polymerization of the haematin solutions used on different days or at different times after preparation.

Combination of haem with globin

Equilibrium studies by Shack & Clark (1947) have suggested that haem (Fe^{2+}) in aqueous solution is dimeric, and it was hoped that the reactions of this derivative with globin might be more suitable for kinetic analysis.

The reaction of $33\text{ }\mu\text{M}$ -haem reduced by the addition of 1 mg. of sodium dithionite/ml. with 1 equivalent of haemoglobin globin gave an initial velocity constant of $3 \times 10^4\text{ M}^{-1}\text{ sec}^{-1}$ at 17° and pH 7.1.

The rate of reaction did not show the marked falling off which is so prominent in the records of Figs. 2 and 3.

Combination of carboxyhaem with globin

In view of the rather more satisfactory results obtained with free haem the reaction with carboxyhaem was examined next. Hill (1926) concluded that this compound has the formula CO-H-X , where H represents the haem nucleus and X may be either another molecule of the complex or a molecule of water. Since then it has generally been

Fig. 3. Mesohaematin combining with myoglobin globin and haemoglobin globin. Haemoglobin globin $33\text{ }\mu\text{M}$, myoglobin globin $33\text{ }\mu\text{M}$, mesohaematin $33\text{ }\mu\text{M}$. Wavelength of observation $397\text{ m}\mu$; 21° ; phosphate buffer, pH 7.1; light path 2.38 mm. O, Myoglobin globin; x, haemoglobin globin. Deuterohaematin combining with myoglobin globin and haemoglobin globin. All conditions were as for mesohaematin. ●, Myoglobin globin; x, haemoglobin globin.

assumed to be monomeric in aqueous solution, a belief confirmed by Smith's (1959) spectrophotometric observations.

It was expected that if carboxyhaem is indeed monomeric the reaction rate observed might more nearly represent the true kinetic possibilities of haem derivatives in combining with globin than that observed with haematin and haem.

Fig. 4. Combination of carboxyhaem with human haemoglobin globin. Four concentrations of carboxyhaem and globin were used, giving concentrations after mixing of 3.2, 1.64, 0.84 and $0.43\text{ }\mu\text{M}$ of each. The reagents were dissolved in borate buffer, pH 9.1, equilibrated with a partial pressure of 200 mm. carbon monoxide in nitrogen. The reaction was followed at $421\text{ m}\mu$ with a light-path length of 2 cm. The observations are shown as points; the continuous lines were computed from equations (1) and (2) by numerical methods and broken lines were calculated from equation (8) with the values derived from Fig. 7. [Broken lines start from lower concentrations of reagents because equation (8) supposes that an equilibrium concentration of haem-globin complex is present immediately upon mixing.] Values of the constants used in obtaining the continuous lines were: $k_1 = 4.75 \times 10^6\text{ M}^{-1}\text{ sec}^{-1}$, $k_2 = 1750\text{ sec}^{-1}$, $k_3 = 375\text{ sec}^{-1}$.

The reaction observed with carboxyhaem was rapid, and an example of the results is given in Fig. 4, where four different sets of initial concentrations of carboxyhaem and globin were used. This experiment showed that the reaction was apparently second order with the lowest concentration examined, and gave a value for the rate constant of $8 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$. This value is among the highest ever to be obtained directly by flow methods, and the reaction is one of the most rapid known involving biological materials. Closer examination of the results has shown that the reaction is complex, and a detailed reaction scheme is proposed in a later section. In order to allow comparisons between experiments carried out under different conditions, the results have been calculated as if the reaction were second order, assuming that the concentrations of reactants were those put in the apparatus rather than those calculated from the observed changes in extinction. The reasons for this appear below.

Effect of pH and of temperature. A detailed study of the effect of pH has not yet been made, but experiments have been carried out at pH 7.1 and 9.1. The results, together with those giving the

effect of temperature on the rate of the reaction, are summarized in Fig. 5, which shows that the observed rate is doubled by raising the pH to 9.1. The temperature coefficient is similar at both pH values and corresponds to an activation energy of about 11 kcal.

Influence of other factors on the observed reaction

Wavelength of observation beam. The greater number of observations have been made with light of $421 \text{ m}\mu$, corresponding to the maximum light absorption of carboxyhaemoglobin. In a few cases the reaction has also been followed by the changes taking place at $407 \text{ m}\mu$, the absorption maximum of carboxyhaem. The observed rates have agreed well as measured at the two wavelengths.

Effect of salt concentration. The rate of combination of carboxyhaem with exhaustively dialysed globin dissolved in distilled water was measured. The haemin was dissolved in a minimum of NaOH solution and reduced under anaerobic conditions with a small excess of dithionite. The quantities of salts used were calculated to give a final ionic strength of less than 1×10^{-4} . After the determination of the combination rate an oxygen-free saturated solution of $(\text{NH}_4)_2\text{SO}_4$ was added to each reagent in quantities sufficient to give a final concentration of 0.1 M . $[(\text{NH}_4)_2\text{SO}_4$ was used because larger numbers of charges are provided per mole than with, say, NaCl]. The rates measured were, at 19° : 'salt-free', 6, and, with salt, $5 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$.

Effect of high concentrations of sucrose. In view of the high rate of the combination reaction, experiments in 40% (w/v) and 60% (w/v) sucrose were carried out. The reaction appeared to be slightly slowed (about 50%) by 40% sucrose and substantially slowed (to one-fifth to one-tenth) by 60% sucrose in experiments at 18° , with carboxyhaem and globin concentrations of $1 \mu\text{M}$ after mixing. As it seemed possible that this effect was due to difficulties in mixing the viscous solutions, a control experiment was carried out in which whale myoglobin was allowed to combine with carbon monoxide. Both the myoglobin and the carbon monoxide were dissolved in 60% (w/v) sucrose which had been prepared in 0.1 M -phosphate buffer, pH 7.1. The reaction was first followed in the stopped-flow apparatus at $440 \text{ m}\mu$, and then equal volumes of the two reagent solutions were intimately mixed and transferred to an observation cell suitable for the flash apparatus of Gibson (1959), and the reaction was followed photochemically as described by Gibson (1956), with light of mean wavelength $440 \text{ m}\mu$. The total changes of extinction and the rate of the combination reaction agreed within 10% by the two methods. The concentration of carbon monoxide was so chosen that the rate expressed as a first-

Fig. 5. Effect of temperature and pH on the observed rate of combination of carboxyhaem with globin. The ordinate gives the logarithm of the apparent second-order velocity coefficient. ●, Observations made with borate buffer, pH 9.1; ○, observations made with phosphate buffer, pH 7.1. Observations were made at $421 \text{ m}\mu$, 2 cm . light-path length. Concentrations of haem and globin after mixing were $1 \mu\text{M}$ each.

order constant in the control experiment was 50 sec.^{-1} , whereas in the carboxyhaem + globin experiments the observed rate in sucrose was only 4 sec.^{-1} . It seems therefore that the effect of sucrose is not due to deficiencies in the stopped-flow apparatus.

Effect of alteration of globin. The effect of alkaline denaturation of the globin was examined in experiments in which a portion of globin solution (0.1 mM), after standing for 10 min. at 18° in the presence of 0.1 N-NaOH , was mixed with 50 vol. of 0.1 M -buffer, pH 10.6, and oxygen was removed with dithionite (0.5 mg./ml.). The reaction with carboxyhaem was found to be even more rapid than that of native globin, the observed rate constant at 18° being $1.6 \times 10^8 \text{ M}^{-1} \text{ sec.}^{-1}$. The occurrence of denaturation was checked by spectroscopic observation of the band at $557 \text{ m}\mu$ formed on reaction of the globin solution with free haem. In view of the very high rates found in all the experiments with carboxyhaem so far described, its reaction with pyridine was next examined. In one experiment in which $3 \mu\text{M}$ -carboxyhaem was allowed to react at pH 7.1 and 18° with 2.5 mM -pyridine, the rate constant found was $330 \text{ M}^{-1} \text{ sec.}^{-1}$. The reaction is pH-sensitive, but was not investigated in detail. Imidazol was similar to pyridine.

Reaction of mesocarcboxyhaem with globin. In a single experiment at pH 9.1 and 20° , of the type shown in Fig. 4, and covering a similar range of concentrations, the observed rate of reaction was found to be $6.5 \times 10^7 \text{ M}^{-1} \text{ sec.}^{-1}$. The experiment was carried out exactly as for protohaem except that observations were made at $409 \text{ m}\mu$, the wavelength of maximum absorption of carboxymesohaemoglobin.

Reaction of myoglobin with carboxyprotohaem. This protein has been less fully studied than haemoglobin globin, but in three experiments it has been found that the mean rate of combination is $2.8 \times 10^7 \text{ M}^{-1} \text{ sec.}^{-1}$ at pH 9.1 and 20° . The temperature coefficient is 2.0 per 10° temperature rise (one experiment), corresponding, as with haemoglobin globin, to an activation energy of about 10 kcal.

Mechanism of the reaction between carboxyhaem and globin

Anomalous observations. Although the course of the reaction appeared to be satisfactorily second order with very low concentrations of carboxyhaem and globin, closer analysis of the data already given shows important inconsistencies. For example, the time course of the reactions illustrated in Fig. 4 differs as between the higher and lower concentrations. In Fig. 6 the reciprocals of the concentrations of carboxyhaem and globin (which were present in equal amounts in this experiment) are plotted against time. If the reaction were

accurately second order, a series of parallel straight lines should be obtained. At the higher concentrations there is a marked curvature at the lower ends of the lines, and the slope of the lowest line is distinctly less than that of the others. Further, the rapid changes in extinction observed in the flow apparatus do not increase in proportion with the concentrations of carboxyhaem and globin. In Table 1, the observed and expected changes at $421 \text{ m}\mu$ are set out, due allowance being made for the dead time of the apparatus (3 msec.). The last column shows that there is a progressive fall in the ratio of the observed to expected change in extinction as the concentration of the reagents is increased.

Two other findings require explanation. The experiments shown in Fig. 6 lead to a velocity constant of about $8 \times 10^7 \text{ M}^{-1} \text{ sec.}^{-1}$, whereas the temperature coefficient derived from Fig. 5 gives an activation energy of about 11 kcal. The corresponding value of the frequency factor is 10^{16} , or about 10^6 times greater than that usually met with in bimolecular reactions. Again, increasing the viscosity 60-fold with sucrose gave a significant

Fig. 6. Treatment of data from Fig. 4 to show deviation from the second-order relation. The ordinate gives the reciprocal of the concentration of carboxyhaem remaining. If the reaction were accurately second order a family of parallel straight lines would be obtained.

Table 1. *Comparison of observed, corrected and calculated changes of extinction*

Extinction changes were (i) observed in flow apparatus from 3 msec. to 5 sec., (ii) corrected for dead time and (iii) calculated from known concentrations of reagents used. Data refer to the experiment of Fig. 4. Figures under 'Corrected' were derived by assuming that the dead time of the flow apparatus is 3 msec. Multiplication of this time by the first-order velocity constant for the reaction being observed gives the natural logarithm of the ratio $\frac{E \text{ at time } 0}{E \text{ at time } 0.003}$. 'Calculated' figures suppose a difference in millimolar extinction coefficients at 421 m μ for carboxyhaem and carboxyhaemoglobin of 120, and are obtained by multiplying this figure by the light-path length (2 cm.) and the concentration of reagents given in column 1.

Globin (μM)	E			Corrected/ Calculated
	Observed	Corrected	Calculated	
3.2	0.413	0.505	0.768	0.658
1.64	0.250	0.293	0.394	0.744
0.84	0.149	0.170	0.202	0.842
0.43	0.085	0.094	0.103	0.913

decrease in the rate of reaction, which would not be expected for a reaction with so high an activation energy. Further, the use of sucrose much increased the change in extinction that could be observed.

Suggested mechanism. It appears that the finding set out in the preceding paragraph can be explained by supposing that the reaction takes place in two stages. In the first of these a complex is formed reversibly between carboxyhaem and globin, whereas in the second the carboxyhaem is bound firmly and irreversibly to the globin. The spectroscopic change corresponding to the conversion of carboxyhaem into carboxyhaemoglobin is assumed to take place when the complex is formed.

These suppositions can be formulated as follows (writing $y = [\text{globin}] = [\text{carboxyhaem}]$; $x = [\text{complex}]$; $k_1 = \text{rate of formation of complex}$; $k_2 = \text{rate of breakdown of complex to carboxyhaem and globin}$; $k_3 = \text{rate of formation of carboxyhaemoglobin from complex}$):

$$\frac{dy}{dt} = k_2x - k_1y^2, \quad (1)$$

$$\frac{dx}{dt} = k_1y^2 - (k_2 + k_3)x. \quad (2)$$

These equations do not have an analytical solution. Numerical values have been obtained by standard desk methods and, on a larger scale, with the help of an electronic computer. There is a close analogy between this mechanism and the ordinary Michaelis-Menten formulation of enzyme action, and some of the standard procedures of enzyme kinetics may be applied to give approximate values of the constants in the equations. If it is assumed that k_1 and k_2 exceed k_3 sufficiently, a plot of $1/v$ against $1/S$ may be used to evaluate k_3 . In this case, the rate of disappearance of substrate (change in y) is measured, rather than the rate of formation of products, and v is defined as the rate

constant for the change in extinction. Adding (1) and (2):

$$\frac{dx}{dt} + \frac{dy}{dt} = -k_3x. \quad (3)$$

On the assumption of equilibrium (i.e. $k_1 + k_2 \gg k_3$),

$$k_1y^2 = k_2x, \quad (4)$$

differentiating (4)

$$\frac{2k_1}{k_2}y \frac{dy}{dx} = 1, \quad (5)$$

substituting in (3)

$$\frac{dy}{dt} = -\frac{k_3y^2}{2y + k_2/k_1}. \quad (6)$$

The pseudo-first-order velocity constant actually measured = $v = -1/y(dy/dt)$ so

$$1/v = \frac{2 + k_2/k_1y}{k_3}. \quad (7)$$

The intercept on the y axis, when $1/v$ is plotted against $1/y$, is thus equal to $2/k_3$, and the slope gives the value of the 'equilibrium' constant k_2/k_1 . The progress curve of the reaction may be calculated with the integrated form of (6):

$$k_3t = 2 \ln(y_0/y_t) + k_2 \frac{(1/y_t) - (1/y_0)}{k_1}, \quad (8)$$

where y_0 and y_t are the amounts of carboxyhaem or globin present at times 0 and t respectively.

Comparison between theory and observation. In Fig. 7 a plot of $1/v$ against $1/y$ shows that a reasonably straight line is obtained for the data of Fig. 4, v being measured in sec.⁻¹ over the interval 3–8 msec. after mixing, and y expressed as the mean concentration of free globin during this interval. The progress curves shown as broken lines in Fig. 4 were calculated from the approximate equation (8). These reproduce both the rates and changes of extinction of the experiment with good agreement.

In Fig. 4 the broken lines lie in every case to the left of the point observed 3 msec. after mixing. This is because (8) does not take into account the time required for the formation of the carboxyhaem-globin complex. This time delay offers a rough means of determining the sum of k_1 and k_2 . In the example of Fig. 4 it is about 0.8 msec. By

Fig. 7. Relation between the reciprocal of the observed pseudo-first-order velocity constant measured over the time interval 3–8 msec. after mixing and the reciprocal of the concentration of carboxyhaem remaining 3 msec. after mixing. This figure illustrates the relation of equation (7), and leads to $k_2/k_1 \approx 0.27 \mu\text{M}^{-1}$, $k_3 \approx 320 \text{ sec.}^{-1}$. For further explanation see text. Data and conditions are from Fig. 4.

Table 2. Comparison between observed and calculated results for the combination of carboxyhaem and globin

Concentration of carboxyhaem was $3 \mu\text{M}$ throughout after mixing borate, pH 9.1, at 18° , $421 \text{ m}\mu$, light path 2.0 cm. Figures under give the change at $421 \text{ m}\mu$ between 3 msec. and 10 sec. after mixing. Rate is the pseudo-first-order velocity constant calculated over the time interval 3–8 msec. after mixing. Calculated values were obtained with $k_1 = 4 \times 10^8 \text{ M}^{-1} \text{ sec.}^{-1}$, $k_2 = 8 \times 10^2 \text{ sec.}^{-1}$, $k_3 = 2 \times 10^2 \text{ sec.}^{-1}$.

Globin (μM)	<i>E</i>		Rate (sec.^{-1})	
	Observed	Calculated	Observed	Calculated
19.5	0.098	0.047	180	177
10.0	0.147	0.102	157	150
5.1	0.223	0.221	102	98
2.6	0.299	0.290	62	61
1.4	0.259	0.130	41	87

numerical computation with (1) and (2), and taking the ratio $k_2/k_1 = 3.8$, it is found that, with

$$k_1 = 4.75 \times 10^8 \text{ M}^{-1} \text{ sec.}^{-1}, k_2 = 1750 \text{ sec.}^{-1}$$

and $k_3 = 375 \text{ sec.}^{-1}$, a good fit to the family of curves of Fig. 4 is obtained. These results are shown as continuous lines in Fig. 4.

A much more stringent test of the assumptions used in these calculations can be obtained by varying the concentration of globin while keeping that of carboxyhaem constant. A part of such an experiment is reproduced in Table 2, which shows the observed and calculated changes of extinction from 3 msec to 10 sec. after mixing, and the observed and calculated rate constants for the change of extinction between 3 and 8 msec. after mixing. The calculated results were obtained by the numerical procedure, with the values given in the table. The results with 2.6 and $5.1 \mu\text{M}$ -globin agree well: with 10 and $19.5 \mu\text{M}$, although the rate constants are in good agreement; the observed changes of extinction are greater than expected. This may be due to the neglect of the mixing time of the flow apparatus, the calculations assuming that complete mixing takes place instantaneously, but is much more likely to be due to the carboxyhaem-globin complex having a lower extinction at $421 \text{ m}\mu$ than carboxyhaemoglobin. With the lowest globin concentration the change of extinction observed is again greater than expected, whereas the rate constant is only about half the calculated value. This result would be observed if, in addition to combining with the specific sites on the globin to give carboxyhaemoglobin, further carboxyhaem molecules could form a carbon monoxide haemochromogen by combination at non-specific sites on the protein. Such a reaction would, of course, only be observed under conditions where the carboxyhaem was in excess, as the specific sites combine so rapidly with carboxyhaem. In principle, this secondary combination could be examined kinetically and its effects allowed for in calculations of the kind shown in Table 2, if this seemed desirable.

Values of kinetic constants under various conditions

Analyses similar to those just described in detail have been made for experiments under various conditions, and approximate values for the constants are suggested in Table 3. The fit to the experimental data was usually as good as that in Fig. 4, and in all cases the determinations were made with equal concentrations of carboxyhaem and globin.

The results show that both the formation of the carboxyhaem-globin complex and its conversion into carboxyhaemoglobin are influenced strongly by pH and temperature, the stability of the complex being increased tenfold by changing the pH

Table 3. *Results of kinetic analysis of the reaction between globin and carboxyhaem under various conditions*

Observations were made with equal concentrations of carboxyhaem and globin in all cases. The concentrations are those after mixing. Light of wavelength 421 m μ , path 2 cm., was used throughout. Further description of the experiments is given in the text.

Concn. (μM)	Observed rate (sec. ⁻¹)	pH	Temp.	k_2/k_1 (μM)	k_3 (sec. ⁻¹)	Special conditions
1.0	11.5	7.1	2°	0.50	23	—
1.0	26	7.1	19	0.36	73	—
1.0	45	7.1	25	0.22	120	—
1.2	46	7	19	0.26	123	Low salt expt.
1.2	30	7	19	0.48	86	Control for low salt expt.
0.44	2.5	7.1	18	10	50	60% Sucrose
0.86	5.2	7.1	18	6	58	60% Sucrose
1.68	8.4	7.1	18	8	57	60% Sucrose
*	*	9.1	19	3.8	375	Values from Fig. 4
1.4	30	9.1	20	1.4	110	Myoglobin globin

* No concentrations or rates are given for the experiment from Fig. 4, as the numbers quoted for the constants are averages from the four individual runs of that experiment.

Fig. 8. Combination of monocyanide haem with haemoglobin globin. The plot shows the reciprocal of the pseudo-first-order velocity constant calculated over the period 3–8 msec. after mixing against the reciprocal of the concentration of monocyanide haem present immediately after mixing. Observations were made at 435 m μ , light-path length 2 cm.; borate buffer, pH 9.1; 20°. Values were estimated as described in the text: $k_2/k_1 = 0.88 \mu\text{M}^{-1}$, $k_3 = 90 \text{ sec.}^{-1}$.

from 9.1 to 7.1, and k_3 is decreased threefold. The effect of these two factors acting in opposition is to give only a small effect of pH on the observed reaction. The effect of temperature on k_3 allows the activation energy for the step controlled by it to be calculated as 12 kcal.: the ratio k_1/k_2 shows Q_{10} 1.4.

The experiments with 60% sucrose suggest that the increase in viscosity affects primarily the ratio k_2/k_1 , which is increased some 20-fold, whereas

there is little, if any, effect on k_3 . The overall reaction is slowed to about one-fifth, and the proportion of the reaction which can be observed is much increased.

A single set of figures, taken from a more extensive experiment, shows that myoglobin globin and carboxyhaem combine in much the same way as haemoglobin globin.

Combination of monocyanide haem with globin

Although the kinetic analysis of the reactions of carboxyhaem accounts successfully for most of the results, other mechanisms may be proposed to explain them. In particular, a reasonable fit can be obtained with equations (1) and (2) on the assumption that $k_2 = 0$. This can be tested with monocyanide haem, where two kinds of experiment are possible. In one, monocyanide haem is allowed to combine directly with globin; in the other cyanide is displaced from dicyanide haem by globin to give cyanide haemoglobin.

A direct combination experiment is illustrated in Fig. 8. The analysis differs in detail from that of the carboxyhaem experiments: it is found that the observed extinction changes do not decrease strikingly as the concentration of haem and globin is increased (compare Table 1 for carboxyhaem), which suggests that the light absorption of the monocyanide haem-globin complex is similar to that of monocyanide haem at 435 m μ , the wavelength at which the observations were made. The abscissa in Fig. 8 corresponds to $1/(x+y)$, with symbols as defined in equations (1) and (2), instead of $1/y$, and v becomes

$$-\frac{1}{(x+y)} \frac{d(x+y)}{dt}$$

in place of

$$-\frac{1}{y} \frac{dy}{dt}$$

The expression analogous to (7) is then

$$\frac{1}{v} = \frac{1}{k_3} \left(1 + \frac{\sqrt{[aK + \frac{1}{4}K^2] - \frac{1}{2}K}}{a + \frac{1}{2}K - \sqrt{[aK + \frac{1}{4}K^2]}} \right),$$

where $K = k_2/k_1$ and $a = x + y$. The intercept when $1/a = 0$ is therefore $1/k_3$ instead of $2/k_3$ as in Fig. 7. The reciprocal plot is no longer strictly linear, and bends down rather sharply in the area covered by the two lowest points in the Figure. Values of k_3 and k_2/k_1 have been obtained algebraically from the data of Fig. 8, and are:

$$k_3 = 90 \text{ sec.}^{-1}, \quad k_2/k_1 = 0.88 \mu\text{M}^{-1}.$$

A further special difficulty requires mention: as Keilin (1949) has shown, it is not possible to separate the two steps of cyanide addition to haem completely, even at alkaline pH, so that the concentration of monocyanide haem cannot be regarded as equal to the total haem concentration. In practice, the solutions used were prepared by adding dilute (0.1 %) KCN solution to a solution of haem in borate buffer, pH 9.1, until the sharp α -band of monocyanide haem in the green appeared at its strongest as judged with a pocket spectroscope. The amount of monocyanide haem was then measured approximately by making a spectrophotometric reading at $552 \text{ m}\mu$, the maximum of the α -band, and using Keilin's extinction coefficient.

Displacement experiments with dicyanide haem and globin

According to Keilin (1949) haem forms two compounds with cyanide which may be written $\text{CN}-\text{H}-\text{H}_2\text{O}$ and $\text{CN}-\text{H}-\text{CN}$, where H represents the haem nucleus. If the dicyanide compound is unable to combine directly with globin, the reaction must proceed through the intermediate formation of monocyanide haem. This monocyanide haem will either recombine with cyanide to reform dicyanide haem or combine with globin to form the postulated monocyanide haem-globin complex. The complex, in its turn, will either form the relatively stable cyanide haemoglobin, or dissociate to re-form monocyanide haem and free globin. Writing dicyanide haem as y , monocyanide haem as z and monocyanide haem-globin complex as x , the reactions are

where G is the concentration of globin. To examine this scheme experimentally the conditions were adjusted to allow the concentrations of CN^- and globin to be regarded as constant, at least in the early stages of the reaction: further, with high concentrations of cyanide and globin the concentration

of monocyanide haem may be regarded as small throughout the reaction and therefore dz/dt can be taken as zero. Then

$$dz/dt = k_4 y + k_2 x - k_5 z [\text{CN}^-] - k_1 z [G] = 0, \quad (9)$$

$$z = (k_4 y + k_2 x) / (k_5 [\text{CN}^-] + k_1 [G]), \quad (10)$$

$$dy/dt = k_5 [\text{CN}^-] z - k_4 y, \quad (11)$$

$$dx/dt = k_1 [G] z - (k_2 + k_3) x. \quad (12)$$

On substituting from (10) in (11) and (12) the course of the displacement reaction can be calculated by numerical methods. For the special case $k_2 = 0$, standard methods give the solution

cyanide haemoglobin =

$$y_0 (A e^{-k_2 t} - k_3 e^{-At} + k_3 - A) / (k_3 - A), \quad (13)$$

where y_0 is the initial concentration of dicyanide haem and

$$A = k_1 k_4 [G] / (k_1 [G] + k_5 [\text{CN}^-]).$$

To compare theory and experiment, determinations of the rate of the displacement reaction have been made by mixing dicyanide haem in equilibrium with various concentrations of cyanide with globin. This reaction is not easy to follow because the spectrum of dicyanide haem is rather similar to that of cyanide haemoglobin. The results are shown in Fig. 9, where the reciprocal of the rate of replacement is plotted against cyanide concentration. The rate of the reaction is seen to depend on the cyanide concentration, a finding which would not be expected if dicyanide haem were able to react directly with globin.

The quantitative comparison of (11) and (12) with experiment requires that all the velocity constants be evaluated. The ratio k_2/k_1 is already available from the experiments of Fig. 8, together with k_3 . The observed rate of reaction with the lowest globin concentration sets a minimum value to k_1 , which is taken to be $5.6 \times 10^8 \text{ M}^{-1} \text{ sec.}^{-1}$. From this, k_2 is calculated to be 640 sec.^{-1} . Although these values are only approximate, the rate of the replacement reaction is more sensitive to their ratio than to their absolute values, especially when k_1 exceeds 10^8 .

The rate of combination of cyanide with monocyanide haem, k_5 , was measured by flash photolysis. It has been found (Q. H. Gibson, unpublished work) that the quantum yield for the conversion of dicyanide haem into monocyanide haem and CN^- ion is high and may be 1, whereas that for the dissociation of monocyanide haem is less than 10^{-4} and may be zero. If therefore a solution of dicyanide haem is flashed, monocyanide haem is formed, which recombines with CN^- ion, giving the

required rate. The velocity has been measured over a range of cyanide concentrations with the results shown in Fig. 10.

The rate of dissociation of dicyanide haem, k_4 , has been measured in two ways: (a) from the displacement reaction with globin shown in Fig. 9; (b) from the reaction with carbon monoxide yielding the compound CN—H—CO (Keilin, 1949). (a) For the reaction with globin, which is irreversible, the situation is exactly analogous to the replacement of oxygen in combination with myoglobin by carbon monoxide. The theory of this method has been fully developed by Gibson & Roughton (1955), who have shown that if the reciprocal of the rate of replacement is plotted against the partial pressure of oxygen in the solution, a straight line results whose intercept is equal to the reciprocal of the velocity constant of the reaction $\text{oxy myoglobin} \rightarrow \text{myoglobin} + \text{O}_2$. In the

present case, if the reciprocal of the rate of displacement of cyanide is plotted against the concentration of cyanide the value of k_4 is obtained. Such a plot is given in Fig. 9.

Exactly similar reasoning may be applied to method (b), where the reaction is

Since this reaction is reversible, and, at the higher cyanide concentrations, is far from complete, the observed rate constants must be corrected to take into account the total amount of exchange occurring. The details of an experiment are given in Table 4 and the reciprocal plot derived from it is given in Fig. 11. The two methods give results in satisfactory agreement with a mean value for k_4 of 22 sec.^{-1} .

Comparison of theory and experiment

The course of the replacement reaction has been calculated from (11) and (12), with the constants quoted, and the results are inserted in Fig. 9. In view of the number of separate experimental determinations involved the agreement is highly satisfactory and strongly supports the suggested mechanism. The time course of the reaction is also interesting, and one example showing the concentrations of dicyanide haem, monocyanide haem-globin complex and cyanide haemoglobin is given in Fig. 12. There is a lag period in the appearance of cyanide haemoglobin, during which the concentration of complex is building up, and the rate

Fig. 9. Formation of cyanide haemoglobin from mixtures of dicyanide haem and haemoglobin globin reacting in the presence of various concentrations of cyanide. The ordinate gives the reciprocal of the observed velocity constant measured over the interval 3–38 msec. after mixing (units, seconds). Initial concentrations: dicyanide haem, $6 \mu\text{M}$; haemoglobin globin, $10 \mu\text{M}$, dissolved in borate buffer, pH 9.1. Observations were made at $440 \text{ m}\mu$, light-path length 2 cm. O, Experimental observations; ●, results of calculations described in the text.

Fig. 10. Combination of cyanide with monocyanide haem. The figure shows the relation between the second-order rate constant for the combination reaction, as determined by flash photolysis, and the concentration of cyanide used in the experiments. Total haem concentration $5 \mu\text{M}$; light-path length 1 cm.; observations were made at $435 \text{ m}\mu$; temperature 20° ; flash energy 200 J.

constant rises over successive time intervals. The experimental observations show a similar acceleration, which is regarded as offering strong confirmation of the correctness of the mechanism proposed. Finally, equation (13) may be used to give a lower limit for k_1 by considering the position where $k_2 = 0$. In order to reproduce the rates of Fig. 9 with $k_2 = 0$, it is necessary to set $k_1 = 4 \times 10^7$. This is too low for the data of Fig. 8, where, at the lowest concentration of monocyanide haem and globin, a value for $k_1 = 1.8 \times 10^8$ would be required, with $k_2 = 0$. On returning to the data of Fig. 9 with $k_1 = 1.8 \times 10^8$, a value of $k_2 = 200 \text{ sec.}^{-1}$ is required. These values will now not fit the data of Fig. 8, as introduction of a finite value for k_2 slows down the combination reaction. In this way it is found that the values for k_1 and k_2 of 5.6×10^8 and 640 sec.^{-1} are minimum values for the combination reaction.

DISCUSSION

The experiments with haematin and globin are interesting chiefly because the reaction is considerably faster than that observed by Theorell & Mæhly (1950) for apoperoxidase and haematin. In spite of the large differences in rate found in individual experiments, the difference between apoperoxidase and haemoglobin is probably significant.

The experiments with carboxyhaem are technically more satisfactory and have allowed a

Fig. 11. Displacement of cyanide from dicyanide haem by carbon monoxide. This figure represents the data of Table 4 and leads to a value of 20 sec.^{-1} for the rate constant of the replacement reaction: dicyanide haem + CO $\rightarrow$ monocyanide carboxyhaem + cyanide. For further details see text and the legend of Table 4. The deviation of the highest point is due to the large correction required because of the back-reaction at this cyanide concentration.

kinetic analysis which accounts for many of the observed features of the reaction: in particular, the combination of a high rate with a high temperature coefficient, as well as the effect of high viscosity. The formation of a complex between the carboxyhaem and globin speeds up the reaction by allowing the final formation of carboxyhaemoglobin to take place whenever the complex acquires sufficient energy to react, instead of requiring a collision to occur at a time when the reactants happen to possess the required energy. In a sense, then, the system may be regarded as a model of enzyme action, illustrating that a reaction may be speeded without a change in activation energy. The point is further emphasized when the results for globin are compared with those

Fig. 12. Continuous lines show the course of the replacement reaction between dicyanide haem and haemoglobin as calculated from equations (11) and (12). Ordinate shows: for the descending curve, the percentage of dicyanide haem remaining; for the ascending curve, the percentage of cyanide haemoglobin that has been formed; for the lowest curve rising to a maximum, the percentage of monocyanide haem-globin complex. Points represent a progress curve of the reaction as observed experimentally under the conditions specified in the legend to Fig. 9 with the time scale altered to show the agreement in form. Values used in obtaining the calculated curve: $k_1 = 5.6 \times 10^8 \text{ M}^{-1} \text{ sec.}^{-1}$, $k_2 = 640 \text{ sec.}^{-1}$, $k_3 = 90 \text{ sec.}^{-1}$, $k_4 = 20 \text{ sec.}^{-1}$, $k_5 = 2 \times 10^6 \text{ M}^{-1} \text{ sec.}^{-1}$. Cyanide concentration, 1 mM ; globin concentration, $10 \mu\text{M}$.

for pyridine and imidazol where the second-order constants are of the order of $10^2 \text{ M}^{-1} \text{ sec}^{-1}$ in both cases: with solutions as dilute as those used in the experiments with globin the rate of combination would have been decreased to about one-millionth.

The rate of the first step in the reaction, corresponding to k_1 in the scheme, is not known with assurance; the values used must be regarded as minimum ones. For carboxyhaem this statement rests on the finding that equilibrium between the complex and the haem and globin is virtually complete in less than 3 msec., the dead time of the stopped-flow apparatus. The experiments with monocyanoide haem support a similar value for the initial combination rate of this compound: in this case additional evidence is available from the correlation of the rates of the direct combination reaction with the rate of displacement of cyanide from dicyanoide haem by globin. Thus in both cases it seems necessary to assume a rate of about $5 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ for the initial step. Although high, such a value is quite possible in view of the investigation by Alberty & Hammes (1958) of the rate at which a diffusion-controlled reaction can take place with a specific site freely accessible from all points in a hemisphere. The effect of decreasing the salt concentration on the ratio k_2/k_1 (Table 3) is also in the direction to be expected from the later investigation of Hammes & Alberty (1959) of electrostatic effects in such reactions. Similarly, the effect of 60% sucrose on the reaction may be explained by supposing that k_1 is much reduced by the increase in viscosity. It is likely that k_2 would also be decreased because, in the viscous solution, a molecule of carboxyhaem which had dissociated from the globin would have a lessened chance of diffusing out of the reaction radius, and so would tend to recombine almost at once with the reaction site. The effect of pH may be discussed in electrostatic terms: as at the most acid pH examined (pH 7.1) the carboxyl groups of the haem will still be almost completely ionized, the effect would be due entirely to change in the ionization of groups in the protein near to the specific site. Unfortunately the range of pH which can be used is small because neither the carboxyhaem nor the globin solution is stable over a wide range.

The specificity of the combination reaction is not high since both myoglobin globin and denatured globin react at about the same rate as native globin; further, the substitution of mesohaem for protohaem does not greatly affect the results. No experiments with other proteins have been made, but since Kiese & Kurz (1955) have found that a concentration of 0.03 mM free protohaem is required for half-saturation of plasma albumin it is evident that globin has an affinity of an altogether higher order.

The work of Rossi-Fanelli *et al.* (1958) has shown that at neutral pH and high ionic strengths (between 0.1 and 0.3) native human globin has an average molecular weight of 41 000 and is thought to consist mainly of molecules with half the molecular weight of haemoglobin. The stability to changes in pH and temperature are also quite different from those of haemoglobin. Rossi-Fanelli *et al.* (1958) have shown that all these differences between globin and haemoglobin disappear after the reconstitution reaction. Since most of the globin molecules are half-sized it is likely that we have been dealing in this paper only with a first step in the reconstitution of haemoglobin and that a complete description of the reaction must include an account of the process by which the molecular weight returns to 68 000. It is intended to follow the later stages of the reaction by a variety of methods, including light-scattering, and rapid thermal methods as well as more conventional kinetic procedures.

The results with monocyanoide haem, which has one more negative charge than carboxyhaem, do not show wide differences from those with carboxyhaem, but as the experiments were all carried out in solutions of high ionic strength, effects due to charge would be minimized. In addition to the difficulties in preparing solutions of monocyanoide haem of known concentration, it was necessary to take into account the instability of cyanide haemoglobin, described by Keilin & Hartree (1955). In confirmation of their work it was found that in the course of a minute or so the spectrum of cyanide haemoglobin was replaced by that of reduced haemoglobin. This change was slower with high cyanide concentrations. In no case did the change interfere with our kinetic determinations, which were always complete in less than 1 sec.

A difficulty in the analysis of the results which has not been fully dealt with in the Experimental section is due to lack of knowledge of the absorption spectrum of the intermediate haem-globin complexes. It has been assumed that at 421 m μ the carboxyhaem-globin complex has the same absorption as carboxyhaemoglobin, but that the monocyanoide haem-globin complex has, at 435 m μ , the same absorption as monocyanoide haem. It is possible, by detailed examination of the changes of extinction in experiments forming part of a series, to arrive at a rough estimate of the true values. It appears that the errors in k_2/k_1 and k_3 due to the assumptions about the absorption coefficient are less than 10%, but the fit in Fig. 4 could be improved by altering the value taken for the extinction coefficient of the complex.

The experiments with dicyanoide haem show that this compound does not react directly with globin. Carboxyhaem does react directly, since the rate of

Table 4. *Displacement of cyanide from dicyanide haem by carbon monoxide*

Dicyanide haem in equilibrium with KCN of the concentration shown in column (1), dissolved in borate buffer, pH 9.1, was mixed with 1 mM-carbon monoxide also dissolved in borate buffer. The extinction change recorded in column (2) was observed at 434 m μ with a path length of 2.38 mm. The 100% value used in calculating column (3) cannot be observed directly: it was obtained by extrapolation of the data of column (2) to zero cyanide concentration. Column (4) gives the rate constant with which the extinction change of column (2) took place. Column (5) gives the rate constant that would have been observed if complete displacement had occurred in each case. Column (6) gives

$$K = [\text{H}(\text{CN})_2] [\text{CO}] / [\text{H}(\text{CN}) (\text{CO})] [\text{KCN}],$$

where H is the haem nucleus.

Cyanide (m) (1)	ΔE (2)	ΔE (as percentage) (3)	Rate constant (sec. ⁻¹) (4)	Rate constant $\times \Delta E$ 100 (5)	Equilibrium constant (6)
0.182	0.026	14.8	36.4	5.4	0.030
0.091	0.055	31.4	31.8	10.0	0.024
0.045	0.090	51.4	26.4	13.6	0.022
0.0018	0.168	96.0	20.0	19.2	0.024
100%	0.175	—	—	—	—

reaction is independent of the concentration of carbon monoxide in solution. This result appears to prove that carboxyhaem is monomeric, since, if it were dimeric with the formula CO—H—H—CO, no reaction could take place with globin until a molecule of carbon monoxide had dissociated from the dimer. This result confirms Smith's (1959) suggestions, based on spectroscopic evidence. The data of Table 4 may be used to calculate the rate of combination of carbon monoxide with monocyanide haem, by the method of Gibson & Roughton (1955). The value found is $1 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$, which compares with the value of $1.6 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ found by Smith (1959) for the combination of carbon monoxide with free haem.

The experiments throw some light on the accessibility of the haem site to carboxyhaem and monocyanide haem; it seems fair to conclude that in the stage of complex formation the haem molecule lies on or near the surface of the globin. It is not possible to say what chemical groups are involved in complex formation, particularly as the spectrum of the complex in one case resembles carboxyhaemoglobin, and in the other, monocyanide haem. Work with a variety of haems (e.g. without carboxyl groups) may help in this respect.

Recently Perutz *et al.* (1960) have completed the task of describing the polypeptide chains of the haemoglobin molecule. Although this work is a landmark in the history of haemoglobin chemistry it does not seem possible to discuss our results in terms of their findings. Globin and haemoglobin differ widely in many ways, and the polypeptide chains in a solution of globin may be arranged differently from those in the oxyhaemoglobin crystal. It is interesting, however, that the similarity between the chains in myoglobin and haemoglobin revealed by X-ray analysis is duly reflected

in similar kinetic behaviour with carboxyhaem and haematin. It can also be supposed that after primary complex formation the secondary process described by k_3 corresponds to a folding of the globin chain around the haem group to give a stable form similar to that described by Perutz *et al.* (1960), but this is no more than a speculation.

SUMMARY

1. Proto-, meso- and deuterio-haematin react with human globin at a rate determined by the properties of the haematin.
2. Carboxyprotohaem reacts rapidly with globin. An intermediate reversible complex is formed with combination and dissociation velocity constants of $5 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ and 1700 sec^{-1} respectively at pH 9.1 and 19°.
3. The complex is converted into carboxyhaemoglobin in a first-order process with rate constant 370 sec^{-1} at 19° and pH 9.1. The activation energy is 12 kcal.
4. The effects of pH, viscosity and temperature on the reaction of carboxyhaem with globin have been examined.
5. Similar results have been obtained with monocyanide haem both by following the direct reaction and by observing the rate of replacement of cyanide in dicyanide haemoglobin by globin.

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Intermediates in the Catalytic Action of Lipoyl Dehydrogenase (Diaphorase)

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Lipoyl dehydrogenase was first discovered in bacteria and shown to be involved in the oxidation of keto acids (Gunsalus, 1954; Reed, 1957). A similar involvement has been shown in mammalian tissues, particularly in the pig-heart α -oxoglutarate-dehydrogenase complex (Sanadi, Langley & White, 1959). Massey (1958, 1960*a*) demonstrated that the lipoyl dehydrogenase of pig-heart muscle is a flavo-protein identical with the flavoprotein which was isolated by Straub (1939) and studied by Savage (1957). This conclusion has been confirmed by Searls & Sanadi (1959), but has also been dismissed (Ziegler, Green & Doeg, 1959). A likely explanation of the inability of Ziegler *et al.* to observe lipoyl-dehydrogenase activity in their diaphorase preparation has, however, been found in the sensitivity of this enzyme to trace metals (Veeger & Massey, 1960), which can abolish the activity towards lipid acid although at the same time it greatly stimulates the activity towards 2:6-dichlorophenol-indophenol, a dye commonly used to assay diaphorase activity. Further studies of this enzyme have shown that two, rather than one, molecules of pyridine nucleotide take part in the reaction:

Lipoic acids + reduced diphosphopyridine nucleotide + H^+ $\rightleftharpoons$ reduced lipoic acids + diphosphopyridine nucleotide

and that a protein dithiol group is formed as an intermediate (Massey & Veeger, 1960).

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Savage (1957) had reported that on the addition of reduced diphosphopyridine nucleotide to diaphorase there was incomplete bleaching of the 450 m μ band of the flavin and an increase in absorption at wavelengths greater than 500 m μ . This observation has been confirmed by Searls & Sanadi (1959). The formation of a red colour with many flavoproteins on the addition of substrates has been reported: e.g. old yellow enzyme (Haas, 1937); reduced diphosphopyridine nucleotide peroxidase (Dolin, 1956, 1957); acyl-coenzyme A dehydrogenases and snake-venom L-amino acid oxidase (Beinert, 1957); diaphorase (Savage, 1957); dihydro-orotic dehydrogenase (Friedmann & Vennesland, 1958); D-amino acid oxidase (Massey & Palmer, 1960). The cause of such a red colour has been the subject of much discussion. In general, the formation of a red colour has been taken to indicate the existence of the enzyme flavin in a half-reduced (or semiquinone or free radical) form, by analogy with the studies on free flavins (Kuhn & Wagner-Jauregg, 1934; Michaelis, Schubert & Smythe, 1936; Beinert, 1956). With reduced diphosphopyridine nucleotide peroxidase, however,

Dolin has presented much evidence that makes it difficult to accept this interpretation of the spectral changes observed with substrates and dithionite. At the moment no generalizations can be made;