are given in Table 2. The amount of hexosamine found in the ox-hide collagen by this method was even smaller than the corrected value obtained directly on the hydrolysate, and the amount found in the citrate-soluble collagen was negligible.

The sugars and sugar derivatives which have been identified in ox-hide collagen are the same as those found in other collagenous tissues, e.g. tendon, but the relative proportions are different and their total amount is less, especially in the citrate-soluble collagen. The negligible amount of hexosamine and the very small and variable amounts of hexoses found in the soluble protein suggest that these may be present as impurities. This is contrary to the views of Kuntzel (1954) and of Grassmann, Endres & Steber (1954) who have found appreciable amounts of hexoses and amino sugars, respectively, which they consider to be an integral part of the citrate-soluble collagen molecule.

SUMMARY

1. Some methods used for the determination of sugars have been examined. Their application to complete protein hydrolysates is discussed.

2. The carbohydrate content of ox-hide collagen and citrate-soluble collagen has been determined.

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Cyanide Compounds of Ferropoxidase and Myoglobin and their Reversible Photodissociation

By D. KEILIN AND E. F. HARTREE
Molteno Institute, University of Cambridge

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The inhibition of peroxidatic activity of tissue extracts by cyanide was already known to Linossier (1898), who was the first to ascribe this activity to special oxidizing enzymes which he named peroxidases. The formation of a reversible and well-defined compound between horse-radish peroxidase and cyanide was noted in the course of spectroscopic studies of purified preparations during which the haematin nature of peroxidase was definitely demonstrated (Keilin & Mann, 1937). The absorption spectrum of this peroxidase-cyanide was found to be of the same pattern as those of the cyanide compounds of methaemoglobin and catalase (Keilin & Hartree, 1951). Since cyanide

combines reversibly not only with the trivalent iron of these three haemoproteins but also with the bivalent iron of haem, haemoglobin and myoglobin, a property which cyanide shares only with nitric oxide (J. Keilin, 1955), it appeared interesting to investigate the possibility of a reaction between cyanide and ferropoxidase.

The objects of this paper are: (1) to show that ferropoxidase, like haemoglobin and myoglobin, forms a reversible compound with cyanide, (2) to define the conditions under which these compounds are formed, (3) to compare their properties with those of other haem compounds, and (4) to demonstrate their reversible dissociation in light. The

photodissociation of these compounds is remarkable, considering that the only haem compounds hitherto known to undergo such dissociation are CO derivatives.

MATERIALS AND METHODS

Peroxidase. Horse-radish peroxidase was the same material as used in previous investigations (Keilin & Hartree, 1951, 1955). The stock solution was 0.46 mM in terms of haematin.

Metmyoglobin. A crystalline product isolated from sperm-whale muscle was kindly supplied by Dr J. C. Kendrew.

Oxyhaemoglobin. A crystalline preparation was obtained from horse blood (Keilin & Hartree, 1935).

Haemin. Crystalline material was prepared from horse blood by the method of Schälfejew (1885).

Methyl carbamylamine. An aqueous solution (0.15 M) was kindly supplied by Dr J. Keilin.

Hydrocyanic acid. A solution was obtained by distilling KCN with excess of m oxalic acid in an all-glass apparatus. The distillate was standardized with 0.1 N-AgNO₃.

Haematin estimations. The above haemoproteins were estimated as haematin by the method described in an earlier paper (Keilin & Hartree, 1951).

Determination of HCN in solutions containing peroxidase. A volume of solution containing $>15 \mu\text{moles}$ HCN was added to 15 ml. m oxalic acid in a small all-glass still and distilled almost to dryness into a 25 ml. volumetric flask containing 1 ml. 0.67 N-KOH. The distillate was diluted to the mark and 1 ml. taken for cyanide estimation by the method of Robbie (1945).

pH Determinations. A micro glass electrode (Hartree, 1952) was used with a Pye Universal pH meter. To obtain complete stability the electrode was surrounded by a cylindrical earthed screen of copper gauze. The required volume of solution was transferred from the absorption cell immediately after the necessary spectrophotometric records had been obtained.

Spectroscopic observations. These were made with a Zeiss microspectroscope ocular fitted to a microscope (Keilin & Wang, 1946).

Spectrophotometry without photodissociation. Solutions were usually contained in stoppered cylindrical silica cells of 1 cm. optical path (see below). By using completely filled closed cells, loss of HCN and autoxidation of $\text{Na}_2\text{S}_2\text{O}_4$ were avoided. Extinctions were measured on a Hilger Uvispek spectrophotometer fitted with a glass prism.

Spectrophotometry with photodissociation. A Beckman DU quartz spectrophotometer was used. It was fitted with the maker's large cell compartment (CC) for absorption cells of up to 10 cm. optical depth. For ease of description of the necessary modifications to CC the following conventions will be observed. The front of CC is that normally faced by the operator and from which projects the cell-carrier shift knob; the back is the wall against which the lamp housing is fixed. 'Right' and 'left' are used in the sense appropriate to the operator's normal position in front of the instrument; i.e. the left side is adjacent to the monochromator and the right side to the photocell housing.

In brief the modifications are as follows: (i) a hole is cut in the rear wall of CC to admit light from a mercury-vapour lamp, via a blue filter to pass the 435-8 μm . line. (ii) The

Beckman cell-carrier is replaced by a swivelling carrier which enables either of two absorption cells to be illuminated by Hg-blue light when it is on the optic axis (OA) of the spectrophotometer. (iii) A filter-carrier is introduced between the cell-carrier and the photocell to carry an orange filter opaque to light passed by the blue filter. (iv) A removable mask, fitted to the left of the cell-carrier, introduces a slit in the optical path so as to reduce the width of the beam emergent from the monochromator. This attachment is used only in conjunction with micro-absorption cells.

(i) The position of the rectangular orifice (ro) to admit the photodissociating light is shown in Fig. 1. A suitable cover was prepared to fit into ro so that measurements could also be made under normal conditions.

(ii) The maker's carrier for cells up to 10 cm. rests, through 3-point contact, on a base plate (bp) which slides on two rods (bpr) as shown in Figs. 2 and 3. This carrier was removed and a new carrier fitted to hold two 1 cm. optical cells of conventional dimensions. The new carrier consists of a brass top plate (tp) carrying two brass disks (d_1, d_2), the centres of which are the same distance apart (31.8 mm.) as the notches in the cell-carrier slide rod (sr). After final adjustment of their positions (see below) these disks were soldered to the top plate. Each disk carries four supporting rods (r) shaped to hold an absorption cell by its four corners and the disks are drilled centrally to take 4-B.A. bolts. The bolt passing through the back disk (d_2) is tapped into the top plate, passes through a curved slot (s_1) in the base plate and engages a second curved slot (s_2) in the floor of CC. A piece of fabric glued to the underside of the floor excludes light. Slot s_1 is in the form of an arc concentric with d_1 while the ends of slot s_2 are beneath the positions of the centres of d_1 and d_2 when the cell-carrier is orientated as in Fig. 3. Thus when the cell-carrier slide rod (sr) is pulled out (i.e. towards the operator), d_1 moves on a straight path parallel to sr while d_2 moves into the position on OA previously occupied by d_1 . Before being soldered to the top plate, d_1 and d_2 are orientated so that the absorption cells held in both come to rest on OA with their optical faces at right angles to OA.

(iii) Figs. 4 and 5 illustrate the filter holder which is placed between CC and the phototube housing. It consists of a copper plate drilled at the corners to take the four long bolts which attach CC and the phototube housing to the monochromator. A small projecting tube of 12.5 mm. square section is placed symmetrically around OA and is held to the copper plate by means of bolts passing through two pieces of vulcanite (v_1, v_2). A 30 mm. diameter hole, on OA, in the plate is concentric with a 35 mm. diameter hole in v_1 . A square hole in v_2 holds the projecting square tube in position. The orange filter (f) is retained in position by means of a rubber ring. To ensure that the apparatus was lightproof it was found necessary to edge the copper plate on both sides with black velvet.

(iv) The mask (m) for use with micro cells consisted of a slit 10 mm. high and 1.5 mm. wide. It was bolted to a strip of Perspex which was attached to the front wall of CC by means of a block of the same material (p).

All parts of the assembly (i-iv) were given a dull black finish.

Optical cells for spectrophotometry. Four types of absorption cell were used: types i and ii in the Hilger spectrophotometer; types iii and iv in the Beckman instrument equipped with the modified cell compartment.

i. The stoppered cylindrical cells had a total volume of 1.1 ml. On account of their small size it was necessary to reduce by about 25% the vertical dimension of the beam emergent from the monochromator. This was effected by means of a thin disk, with a central hole 7.5 mm. diameter, attached concentrically to a Perspex collar made to fit

it was then necessary to fit the cell compartment with a light-proof hood.

ii. To measure the intense Soret band of haemoproteins, solutions examined at 1 cm. optical depth in the visible region were transferred to 1 mm. demountable cells (Hilger and Watts Ltd.).

Fig. 1

Fig. 2

Fig. 4

Fig. 6

Scale for
Figs. 1-5

Fig. 3

Figs. 1-5. Modifications to the Beckman spectrophotometer cell compartment, as supplied for cells up to 10 cm. in length, to permit illumination of absorption cells from the side with light from a mercury vapour lamp. 1, front view of cell compartment; 2, view along optic axis from the right, i.e. from the position occupied by the photocell; 3, plan of cell compartment (front of compartment to the right); 4, plan (sectional) of holder for orange filter; 5, side view of filter holder.

Fig. 6. Microcell (as supplied with the Hilger Spekker absorptiometer) in a carrier designed to be interchangeable with the conventional type of 1 cm. Beckman cell in the modified cell-carrier (Figs. 2, 3).

closely into the circular orifice via which the emergent beam reaches the absorption cells. The solid stoppers of these cells were made interchangeable with tubes carrying Thunberg-type hollow stoppers in order that solutions could be equilibrated with various gas mixtures before addition of $\text{Na}_2\text{S}_2\text{O}_4$ placed in the hollow stopper. A special cell-carrier was made to hold these cells. When they were used with the hollow-stoppered extensions, the latter projected above the level of the cell-compartment lid and

iii. Conventional type Beckman cells, $10 \times 10 \times 44$ mm. (int.), were made up with clear glass in place of the usual frosted glass side walls. The mask *m* was removed when these cells were used.

iv. Micro cells as supplied with the Hilger Spekker absorptiometer were used in conjunction with the mask *m*. The internal dimensions of the cells are 19 mm. high $\times$ 10 mm. (optical path) $\times$ 2.5 mm. wide. The corresponding external dimensions are 22, 16, 8.5 mm. These cells were

placed in individual carriers (Fig. 6) which were about 1 mm. narrower than the spaces between the rods *r*. A pair of notches on the base of the holder facilitated handling the cell plus carrier with forceps. By means of plastic shims the positions of the cells with respect to the rods were adjusted so that the cells were exactly on OA. After this had been done it was convenient to change solutions in the cells without removing them from CC.

Light filters. The blue filter was 0.05 M-CuSO₄ in 2.4 N-NH₄OH containing 0.0056 M 3:5-dinitrobenzoic acid which reduces u.v. transmission. The solution, which was stable for 3 days, was contained in a cell of 2.7 cm. optical depth in close contact with the orifice *ro*. The transmission for the 435-8 mμ. line was 69 %.

The orange filter consisted of 0.24 mm. Cobex sheet no. 5028 (BX Plastics Ltd., Higham Station Avenue, London, E. 4). This has a transmission of 72 % at 565 mμ. but is opaque below 520 mμ.

Transmission curves for both filters are given in Fig. 7. At wavelengths between 800 and 1000 mμ. both filters are transparent, but stray light within this region introduced no errors in the present experiments since only the u.v.-sensitive phototube of the Beckman instrument was used.

Fig. 7. Percentage transmission of light filters: ---, Cobex 5028 (0.24 mm.); —, 0.05 M-CuSO₄ in 2.4 N-NH₄OH (27 mm.); ---, 0.05 M-CuSO₄ in 2.4 N-NH₄OH, + 0.0056 M 3:5-dinitrobenzoic acid (27 mm.).

Units of light absorption. *E* (extinction, optical density) = $\log_{10}[I_0/I]$, where *I*₀ and *I* = intensities of incident and transmitted light. *ε*_{mμ} (millimolecular extinction coefficient) = *E*/[concentration (mm) × optical depth (cm.)]. The optical depth was unity in all experiments, except where otherwise indicated.

Abbreviations used

per ³	=ferriperoxidase
per ²	=ferroperoxidase
per ³ CN	=cyanide derivative of ferriperoxidase
per ² CN	=cyanide derivative of ferroperoxidase
per ² CO	=carbon monoxide ferroperoxidase
per ² CH ₃ .NC	=methyl carbylamine derivative of ferroperoxidase
Methb	=methaemoglobin
MethbCN	=cyanide derivative of methaemoglobin
Hb	=haemoglobin (deoxygenated)

HbCO	=carbon monoxide haemoglobin
HbCN	=cyanide derivative of haemoglobin
HbCH ₃ .NC	=methyl carbylamine derivative of haemoglobin
Metmb	=metmyoglobin
MetmbCN	=cyanide derivative of metmyoglobin
Mb	=myoglobin
MbCO	=carbon monoxide myoglobin
MbCN	=cyanide derivative of myoglobin

It is not intended that the abbreviations used for cyanide derivatives should indicate that the cyanide ion rather than free HCN is the reactive species.

All experiments described in this paper were carried out at 19–21°.

RESULTS

Direct spectroscopic study of ferriperoxidase cyanide (per³CN)

Formation of per³CN. The formation of per³CN and its photodissociation were first observed by direct examination with a microspectroscope of per³ treated with cyanide and sodium dithionite (Na₂S₂O₄). The study of these reactions was carried out in 7-mm. diameter tubes with fused optical glass bases, which were placed over the condenser lens of a microscope stage, the substage illumination being supplied by an 8 v, 48 w microscope lamp. The advantages of this arrangement (Keilin & Wang, 1946) are that it requires very little material, that the observations are simple and rapid and that light absorption by the pigment is not affected by dilution of the tube contents, i.e. the intensities of the absorption bands are not altered if there is no dissociation and if the diluting fluid does not react with the pigment.

The tube normally received about 0.1 ml. stock solution of peroxidase diluted with 0.5 ml. 0.1 M borate buffer (pH 9.1–9.5). At this and even at higher pH the absorption spectrum of per³ is still of the acid methaemoglobin type showing bands at 640 and 500 mμ. On addition of a little Na₂S₂O₄ the colour of the solution turns from brown to red and the above absorption spectrum is replaced by that of per² showing a wide strong band at about 558 mμ. and a weaker narrow band at 595 mμ. On treating this solution with HCN (final concentration 0.01 M) the spectrum of per² is replaced by that of per²CN which is of the haemochromogen type with two strong absorption bands: α- 565 mμ. and β- 535 mμ., the α-band being stronger and sharper than the β-band. Similar results are obtained if the order of addition of Na₂S₂O₄ and HCN is reversed. In this case per³ treated with HCN gives per³CN showing a wide strong band at about 542 mμ. and a narrow feeble band at 582 mμ. On addition of Na₂S₂O₄ this spectrum is replaced by that of per²CN. These experiments can be carried out if necessary in even smaller optical tubes of 5 mm.

diameter which require only 0.05 ml. peroxidase diluted with 0.2 ml. buffer.

A decrease in pH causes the characteristic absorption bands of per^2CN to fade and at pH 5.0–5.5 they disappear completely. A finding of special interest is that the addition of cyanide to per^2 at this pH leads to the formation of per^2CN despite the presence of $\text{Na}_2\text{S}_2\text{O}_4$. This was demonstrated as follows.

(a) Stock peroxidase (0.1 ml.) was diluted with 0.5 ml. 0.2M acetate buffer, pH 5, and treated with $\text{Na}_2\text{S}_2\text{O}_4$, when per^2 was immediately formed. After addition of HCN (final concentration 0.01M) the absorption spectrum of per^2 gradually changed into that of per^2CN .

(b) Using the same quantities of reagents, peroxidase at pH 5 was treated with HCN, when the spectrum of per^2CN appeared immediately. Addition of $\text{Na}_2\text{S}_2\text{O}_4$ now had virtually no effect on the spectrum.

(c) At the end of experiments (a) and (b) the solutions were treated with CO. In both cases the spectrum of per^2CN was rapidly replaced by that of per^2CO .

The above experiments show that at the acid pH per^2 is in equilibrium with a small amount of per^3 . HCN and CO, which have great affinities for the trivalent and bivalent iron respectively, shift the equilibrium either towards per^2CN or towards per^2CO .

The facts (i) that per^2CN cannot exist at pH 5.0 but rapidly changes to per^3CN even in presence of $\text{Na}_2\text{S}_2\text{O}_4$ and (ii) that $\text{Na}_2\text{S}_2\text{O}_4$ is still efficient as a reducer of free per^3 at the same pH suggested a further study of these reactions in the complete absence of oxygen. For this purpose experiments were carried out in two modified Thunberg tubes, each provided with two hollow stoppers as shown in Fig. 8 (Keilin & Hartree, 1947). The reagents were pipetted into the three compartments A, B and C of the two tubes as shown in Table 1 and the air in the tubes was replaced by O_2 -free nitrogen. The changes that occurred on mixing the contents were followed spectroscopically.

In Thunberg tube 1 per^3CN was formed immediately on mixing A and B, the pH of the solution being 5.7. After addition of C the spectrum of per^3CN remained practically unchanged and showed only traces of per^2CN . In Thunberg tube 2 the spectrum of per^2CN appeared rapidly on mixing A and B, the pH of the solution being 9.1. After addition of C the spectrum of per^2CN was replaced almost completely by that of per^3CN . The pH of the solution was now 5.6. On opening both tubes the traces of per^2CN rapidly disappeared and a pure per^3CN spectrum was observed.

Photodissociation of per^2CN . During the earlier part of our work on per^2CN the spectroscopic observations frequently revealed marked inconsistencies. Even when conditions were standardized at the apparent optimum for formation of per^2CN

the compound was sometimes incompletely formed and even appeared to decompose rapidly while it was under observation. However, it was found that when a solution in which this rapid dissociation had been observed was left standing on the bench for a short time the per^2CN bands became stronger but would fade again while the solution was being examined under the microspectroscope. Furthermore, this reversible dissociation could be made to occur repeatedly.

Fig. 8. Thunberg tube with two hollow stoppers (see Table 1).

Table 1. Contents of the three compartments A, B and C of two special Thunberg tubes (see Fig. 8)

	Tube 1	Tube 2
A	0.4 ml. peroxidase 2.0 ml. 0.1M borax 0.5 ml. 0.1M- H_3BO_3 0.4 ml. M- H_3PO_4	A 0.4 ml. peroxidase 2.0 ml. 0.1M borax 0.5 ml. 0.1M- H_3BO_3 5 mg. $\text{Na}_2\text{S}_2\text{O}_4$
B	0.05 ml. M-KCN	B 0.05 ml. M-KCN
C	5 mg. $\text{Na}_2\text{S}_2\text{O}_4$	C 0.4 ml. M- H_3PO_4

The only plausible explanation of these findings was that per^2CN is light-sensitive in the same sense as many CO-haem derivatives, and that the illumination reaching the solution via the substage condenser, but not the diffuse light in the laboratory, was effective for dissociation. This view was

confirmed in several ways. Thus the dissociation that occurred while the solution was on the microscope stage could be prevented if the light was either reduced in intensity or intercepted by an orange filter which was opaque to the blue region of the spectrum where per^2CN shows a strong γ -band. On the other hand, a more complete, although still reversible, photodissociation could be demonstrated by placing the solution in an optical cell

tion was negligible and the spectrum illumination was thus scarcely modified when the Point-o-lite was switched on. Reversibility of dissociation could be demonstrated either by interrupting the side illumination or by interposing an orange filter.

These experiments prepared the ground for a quantitative spectrophotometric study of per^2CN and especially of its photodissociation.

Fig. 9. Absorption curves of peroxidase and its derivatives. 0.085 mM peroxidase; 25 mM- $\text{Na}_2\text{S}_2\text{O}_4$; 8 mM-HCN for per^2CN ; 80 mM-HCN for per^3CN . 1 cm. cells used for visible region, 1 mm. cells used for Soret region (400–460 $\text{m}\mu$). Numbers in brackets refer to pH of solutions.

(horizontal dimensions 13×2.5 mm.). When this cell was illuminated from below by light passing through the orange filter, no dissociation was observed. However, when the solution was simultaneously illuminated from the side by means of a 100 candle-power Point-o-lite lamp (beam parallel to the 2.5 mm. dimension) the bands of per^2CN faded almost completely, being replaced by those of per^2 . Since the solutions used were perfectly clear, scattering of the horizontal beam within the solu-

Quantitative study of ferroperoxidase-cyanide

Absorption spectra of per^2CN and related compounds. The study of per^2CN required quantitative spectrophotometric data on the compounds that might appear under the conditions of its formation. These compounds are per^2 , per^2CN and per^3 . Their absorption curves, together with that of per^2CN both in the visible and Soret regions, are represented in Fig. 9. The absorption spectrum of per^3

shows no significant changes within the pH range 5–9: at higher pH the alkaline form appears (Keilin & Hartree, 1951). At a cyanide concentration of 8 mM the curve of per^3CN remains practically unchanged within the pH range 4.6–9.3, while that of per^2 is constant within the range 7.4–9.0 and shows only very little variation within the wider range 5.0–10.5.

Fig. 10. Absorption curves of peroxidase after treatment with HCN and $\text{Na}_2\text{S}_2\text{O}_4$ at various pH (formation of per^3CN). 0.085 mM peroxidase; 25 mM- $\text{Na}_2\text{S}_2\text{O}_4$; 7 mM-HCN; 1 cm. cells. Lines i–vi, peroxidase + $\text{Na}_2\text{S}_2\text{O}_4$ + HCN in the following buffers: i, phosphate pH 9.03; ii, borate pH 9.50; iii, borate pH 6.44; iv, phosphate pH 6.83; v, borate-phosphate pH 6.20; vi, borate pH 10.63. ---, per^3CN ; ----, per^2 (last two curves from Fig. 9).

Effect of pH on the formation of per^2CN . Variations in absorption of 0.085 mM peroxidase containing 8 mM-HCN and ~ 25 mM- $\text{Na}_2\text{S}_2\text{O}_4$ in different buffers are shown in Fig. 10 (full lines). These curves, with the exception of curve vi obtained at very high pH, exhibit four distinct isobestic points which show that they represent mixtures of only two components. Furthermore, these isobestic points lie on the curve of per^3CN

and not on that of per^2 . Thus, despite the presence of $\text{Na}_2\text{S}_2\text{O}_4$, the two components are per^3CN and per^2CN .

A complete record of experiments on the effect of pH is given in Fig. 11 which shows the variations of $\epsilon_{565}^{\text{mM}}$ (i.e. at the maximum of the α -band of per^2CN). The optimum pH for the formation of this compound is about 9.0. At pH > 10.5 no cyanide compound is formed and the solution shows the spectrum of per^2 . As the pH is lowered from 8 to 5 an increasing proportion of per^3CN is present, although at a given pH its proportion is somewhat higher in phosphate than in borate. These results confirm the visual spectroscopic findings described above.

Fig. 11. Variations in $\epsilon_{565}^{\text{mM}}$ (α -maximum of per^2CN) of peroxidase, in presence of HCN and $\text{Na}_2\text{S}_2\text{O}_4$, in different buffers. Experimental conditions as in Fig. 10. Roman numerals correspond to the curves of Fig. 10. With 7 mM-HCN: $\circ$, borate buffer; $\bullet$, phosphate buffer; $\square$, borate-phosphate buffer. With 70 mM-HCN: $\square$, borate buffer. Horizontal lines indicate values of $\epsilon_{565}^{\text{mM}}$ for per^3CN and per^2 .

Effect of HCN concentration on the formation of per^2CN . Variations in cyanide concentration under standardized conditions (0.085 mM per^2 ; 0.08 M borax; 25 mM- $\text{Na}_2\text{S}_2\text{O}_4$; pH 9.15 ± 0.15) gave rise to the family of curves shown in Fig. 12. The isobestic points indicate that the intermediate curves represent mixtures solely of per^2 and per^3CN . From the formation of 50% per^3CN at 1.4 mM-HCN a dissociation constant of 1.3×10^{-3} M can be derived. (This calculation is based upon total cyanide: not cyanide ion or undissociated HCN.) In contrast to this result, per^3CN formation was shown to be virtually complete at all HCN concentrations quoted in Fig. 12 and as much as 85% complete when HCN was as low as 0.085 mM, i.e. equimolar with peroxidase. This corresponds to a dissociation constant of $\sim 2 \times 10^{-6}$ M.

The band maxima obtained with per^2 in presence of 70 mm-HCN (Fig. 12) were not modified by increasing the cyanide concentration and therefore the absorption spectrum of per^2CN can be characterized as α -565.5 $m\mu$. ($\epsilon_{\text{max}}=18.5$); β -535 $m\mu$.

Fig. 12. Absorption curves of ferroperoxidase in presence of different concentrations of HCN. 0.085 mm peroxidase; 25 mm- $\text{Na}_2\text{S}_2\text{O}_4$; solutions made up in 0.1N borax (pH 9.15 $\pm$ 0.15); 1 cm. cells. Cyanide concentrations (mm) as follows: i, 70; ii, 7; iii, 1.4; iv, 0.7; v, 0.07; vi (from Fig. 9), no HCN.

($\epsilon_{\text{max}}=13.6$). Measurements on the same solution in a 1 mm. cell indicated γ -432.5 $m\mu$. ($\epsilon_{\text{max}}=135$). The complete curve is given in Fig. 9.

Relative affinities of CO and HCN for per^2 . Ferroperoxidase exhibits a high affinity for CO and the characteristic two-banded spectrum (Keilin & Mann, 1937; Keilin & Hartree, 1951) appears immediately when peroxidase is treated with CO in presence of $\text{Na}_2\text{S}_2\text{O}_4$. Compound formation was virtually complete when 0.085 mm per^2 was equilibrated with N_2 containing 5% CO, under which conditions the concentration of free CO in solution is 0.05 mm.

The partition constant between HCN and CO was determined in the cylindrical absorption cells fitted with the Thunberg-stoppered extensions. One cell (a) received 0.2 ml. stock peroxidase, 0.4 ml. 0.1M-HCN and 0.8 ml. 0.1M borax. The stopper received about 5 mg. $\text{Na}_2\text{S}_2\text{O}_4$ and the assembly was evacuated and filled three times with a 1:3 CO- N_2 mixture. After equilibration of the contents E^{565} was measured. A second cell (b) was filled similarly, but with 0.4 ml. 0.2M-HCN and a 1:9 CO- N_2 mixture, and E^{565} determined. Since considerable loss of HCN occurred during the evacuations the cyanide concentrations were determined by the method of Robbie (1945) at the end of the experiment. From the known values of E^{565} for per^2CO and per^2CN values of the partition constant

$$K = \frac{[\text{per}^2\text{CO}] [\text{HCN}]}{[\text{per}^2\text{CN}] [\text{CO}]}$$

were determined (Table 2). The results indicate that the affinity of per^2 for CO is about 2500 times greater than its affinity for cyanide.

Photodissociation of per^2CN

Effect of light on the equilibrium of the reaction between per^2 and HCN

Experiments in conventional 1 cm. cells. A solution (y) of per^2CN was made up to contain 0.0184 mm peroxidase and 8.4 mm-HCN in 0.1M borax, pH 9.01. A second solution (x) was identical with y but the HCN was omitted (Table 3). The blank

Table 2. Determination of the partition constant of CO and HCN for ferroperoxidase from measurements of E^{565}

For fillings of cells (a) and (b) (type i with Thunberg tube extensions) see text. K represents the relative affinities of CO and HCN for ferroperoxidase. Temp. = 20°.

Per^2 derivative	E^{565}	Free CO in solution* (mm)	Total cyanide† (mm)	Final pH	$K = \frac{[\text{per}^2\text{CO}] [\text{HCN}]}{[\text{per}^2\text{CN}] [\text{CO}]}$
per^2CO (excess of CO)	0.740	—	—	—	—
(a)	0.755	0.25	15.9	8.9	2400
(b)	0.793	0.10	26.6	8.5	2700
per^2CN (excess of HCN)	1.343	—	—	—	—

* Calculated from solubility of CO in water at 20°.

† Estimated at the end of the experiment.

Table 3. Photodissociation at pH values 9.01 and 7.3 of per^2CN

Peroxidase, 0.0184 mM; $\text{Na}_2\text{S}_2\text{O}_4$, 25 mM. E^{540} measured before addition of $\text{Na}_2\text{S}_2\text{O}_4$. x , per^2 ; y , $\text{per}^2 + 8.4 \text{ mM-HCN}$; z , $\text{per}^2 + 1.0 \text{ mM-HCN}$. *a*, Experiments in 1 cm. cells of conventional dimensions; *b*, experiment in 1 cm. microcells. L, Hg-blue light; D, darkness.

	HCN (mM)	Borate buffer pH 9.01				Phosphate buffer pH 7.3	
		E^{565}	E^{565} after reduction		% per^2CN		% per^2CN
			D	L	D	L	
(a) 3 ml. x	0	—	0.204	0.203	—	—	—
3 ml. $x + 0.1 \text{ ml. } y$	0.28	—	0.238	0.214	26	10	9
3 ml. $x + 0.4 \text{ ml. } y$	0.99	—	0.288	0.238	60	26	35
3 ml. $x + 0.9 \text{ ml. } y$	1.94	—	0.309	0.254	75	37	44
3 ml. y	8.4	0.212	0.330	0.285	93	59	60
(b) z	1.0	—	0.293	0.224*	62	15	—
Calc. E for 100% per^2CN	—	—	0.348	—	—	—	—

* Corrected for stray-light error of 0.002 determined with solution x .

cell of the spectrophotometer contained 0.1M borax. The extinction of y was measured at 540 $\text{m}\mu$. (E_{max} for per^2CN) in order to check the concentration of haemoprotein and to calculate E^{565} for 100% per^2CN formation. All solutions were then treated with $\text{Na}_2\text{S}_2\text{O}_4$ (about 4 mg./ml.) and the extinction at 565 $\text{m}\mu$. was determined (light and dark) for x , y and mixtures of x and y . Solutions containing the same concentrations of peroxidase, HCN and $\text{Na}_2\text{S}_2\text{O}_4$ were made up in phosphate buffer, pH 7.3, and examined in the same way. At this pH the affinity for HCN is lower but a similar photosensitivity was observed (Table 3*a*).

Experiments in microcells. An experiment at pH 9.01, using the microcells (solution z), in which the path for the Hg-blue light is reduced to 2.5 mm., showed an increased photosensitivity of per^2CN at a cyanide concentration of 1.0 mM. In such an experiment it was found that 62% compound formation in the dark was reduced to 15% in blue light (Table 3*b*).

Rates of association and dissociation in the per^2 -HCN reaction

It was observed during the preceding experiments that the formation and dissociation of the cyanide compound during intermittent illumination did not occur very rapidly. While dissociation was always complete in 20 sec., the association in darkness required more than 30 sec. The following methods were devised for determining, with the apparatus described above, the dissociation and association curves, i.e. the curves representing the changes with time of the proportion of free and combined per^2 (Fig. 13). A solution of per^2CN made up as y (above) but containing 1.71 mM-HCN was used to study the processes of (i) dissociation and (ii) association. Absorption cells of standard dimensions were used.

Fig. 13. Process of association in dark and dissociation in Hg-blue light of per^2 and HCN. Experimental solution consists of 0.1M borax containing 0.0184 mM peroxidase, 1.71 mM-HCN and 25 mM- $\text{Na}_2\text{S}_2\text{O}_4$; pH 9.01; Temp. = 18°; standard size 1 cm. cells. For procedure see text. The points $\bigcirc$ and $\square$ represent % CN compound at equilibrium in dark and light respectively.

(i) The sequence of operations in measuring rates of dissociation is as follows: the two extreme steady values E_D (in darkness) and E_L (in Hg-blue light) are determined at 565 $\text{m}\mu$. The extinction dial of the Beckman spectrophotometer is set at some intermediate E value and the following operations are carried out. ('Cell-carrier in' indicates that the blank cell is in the monochromator beam: 'cell-carrier out' indicates that the pigment cell is in the beam.)

a, Hg lamp off, switch at 'check'; *b*, cell-carrier in, adjust dark current; *c*, Hg lamp on, open shutter; *d*, centre galvanometer needle with slit and sensitivity controls; *e*, Hg lamp off, cell-carrier out, switch to '1'; *f*, Hg lamp on, simultaneously start timer; *g*, as galvanometer needle crosses zero line, stop timer; *h*, close shutter, then *a* (above).

Repetition of *a-h* at several pre-set intermediate *E* values, which can be converted into percentage of per^2 combined with HCN, gives the necessary data for construction of the dissociation curve. Since association in darkness is rather slow an interval of 1 min. should be allowed after each sequence. The results of such measurements are shown in Fig. 13. Several measurements of dissociation were made for each value of *E* and the scatter of the time intervals is represented in each case by a horizontal line.

(ii) The association reaction is recorded in the following way: with the extinction dial set at intermediate values the following operations are carried out.

a-e. As above, except that the Hg lamp remains off throughout (i.e. the normal operational procedure); *f*, Hg lamp on until galvanometer needle ceases to move to the left; *g*, Hg lamp off, simultaneously start timer; *h*, as galvanometer needle crosses zero line, stop timer; *i*, Hg lamp off, close shutter, then *a* (above). The association curve is constructed in the same way as the dissociation curve (Fig. 13).

effect of Hg-blue light on the measured E^{540} for per^2 indicated that no stray light reached the photocell. On the other hand, measurements in darkness and in light on the two solutions and on their mixtures (Table 4*c, d*) indicated a distinct photodissociation of per^2CO .

Myoglobin cyanide (MbCN)

Spectroscopic observations. It has been shown by several workers that cyanide reacts with the bivalent iron of haemoglobin and myoglobin, forming rather unstable compounds (Anson & Mirsky, 1939; Stitt & Coryell, 1939; Kiese & Kaeske, 1942). These reactions were recently re-examined and compared with those of cyanide and free haems (J. Keilin, 1955). In the course of the present study we were able to confirm that HbCN and MbCN can easily be obtained by addition of $\text{Na}_2\text{S}_2\text{O}_4$ to the corresponding ferric forms (MethbCN and MetmbCN). While HbCN can be obtained only as a very unstable complex which rapidly liberates Hb, MbCN is sufficiently stable for studies similar to those already described for peroxidase.

Table 4. Photodissociation of per^2CO

Ferropoxidase solution: 0.0184 mm in 0.1M borax containing 25 mM- $\text{Na}_2\text{S}_2\text{O}_4$. Measurements made as in Table 2 at 540 m μ ; 1 cm. cells. L, Hg-blue light; D, darkness. Temp. = 20°.

Treatment of per^2 solution	CO (mm)	E^{540}		% per^2CO	
		D	L	D	L
(a) Saturated with N_2	0	0.148*	0.148	—	—
(b) Saturated with CO	1.0	0.218*	0.210	100	89
(c) 1 vol. <i>b</i> + 14 vol. <i>a</i>	0.066	0.213	0.192	93	63
(d) 1 vol. <i>b</i> + 89 vol. <i>a</i>	0.011	0.180	0.169	46	30

* Extinctions of per^2 and per^2CO .

Photodissociation of per^2CO

The procedure which enabled us to study quantitatively the photodissociation of per^2CN is less suitable for a similar study of per^2CO . This is mainly due to the low solubility of CO, the dissolved concentration of which, even in a solution equilibrated with pure CO, is about 1 mm, which is only five times as high as the concentration of per^2 required for accurate recording of density changes. However, the above method can be used even at lower CO concentrations for a qualitative demonstration of the light sensitivity of per^2CO . For this purpose peroxidase was diluted with 0.1M borax to 0.0184 mm, reduced with 0.025M- $\text{Na}_2\text{S}_2\text{O}_4$ and the solution divided into two parts, *a* and *b* (Table 4). Solution *a* was saturated with N_2 and contained free per^2 , while solution *b* was saturated with CO and contained fully formed per^2CO . The two compounds show a marked difference in extinction at 540 m μ . The lack of

Table 5. Effect of pH on the reaction of Mb with HCN

0.04 mM metmyoglobin, M-KCN and 0.05M- $\text{Na}_2\text{S}_2\text{O}_4$ present in all cases. pH values below 9 obtained by addition of H_3PO_4 ; pH values above 9 by addition of KH_2PO_4 . Results with the former 'buffers' are uncertain owing to volatility of HCN. Extinction measured in stoppered 1 cm. cells.

pH	$\epsilon_{\text{mM}}^{566}$	% MbCN
6.79	16.4	79
7.41	16.1	75
8.51	17.1	87
9.28	18.3*	100
9.59	18.1	98
9.77	17.9	96
9.90	17.4	90
10.08	16.0	74
10.28	14.9	62
10.70	12.3	31

* This was the highest value of $\epsilon_{\text{mM}}^{566}$ that could be obtained and was therefore taken to represent the extinction coefficient of MbCN. $\epsilon_{\text{mM}}^{566}$ for Mb was found to be 9.60 (Table 6).

There is considerable disagreement among the earlier authors on the absorption spectra of both HbCN and MbCN. For the reason already stated we have confined our attention to MbCN and by microspectroscopic tests have established that the MbCN spectrum is of the haemochromogen type and very similar to that of per²CN. The complete formation of the compound requires a very high cyanide concentration and a fairly high pH (~9).

Quantitative spectrophotometric studies. The influences of pH and of cyanide concentration on the

formation of MbCN are demonstrated in Tables 5 and 6. The height of the α -band was maximal at pH 9.3 and at a cyanide concentration of ≤ 1 M. The highest value of ϵ_{566}^{566} , 18.3, was taken to represent 100% compound formation. The absorption curve obtained under these conditions, as shown in Fig. 14, has the following characteristics: α -566 m μ . ($\epsilon_{\text{max}}=18.3$); β -535 m μ . ($\epsilon_{\text{max}}=13.8$); γ -434 m μ . ($\epsilon_{\text{max}}=145$). Measurements at pH < 8 gave unreliable results even in the stoppered cells owing to the volatility of HCN.

Table 6. *Effect of HCN concentration on the reaction of Mb with HCN*

Solution *x*: 0.04 mM metmyoglobin, M-KCN, 0.4 M-KH₂PO₄. Solution *y*: 0.04 mM metmyoglobin in water. After filling the stoppered 1 cm. cells with mixtures of *x* and *y*, Na₂S₂O₄ (~0.05 M) was added. In absence of *x* (last line of Table) 10 mg. K₂CO₃ were added to maintain the solution weakly alkaline.

		pH	HCN (M)	ϵ_{566}^{566} mm	% MbCN
(i)	1.1 ml. <i>x</i>	9.29	1	18.3	100
(ii)	0.9 ml. <i>x</i> + 0.2 ml. <i>y</i>	9.29	0.82	17.6	92
(iii)	0.7 ml. <i>x</i> + 0.4 ml. <i>y</i>	9.27	0.64	16.9	84
(iv)	0.5 ml. <i>x</i> + 0.6 ml. <i>y</i>	9.22	0.46	15.1	64
(v)	0.3 ml. <i>x</i> + 0.8 ml. <i>y</i>	9.15	0.27	12.6	35
(vi)	1.1 ml. <i>y</i> (Mb)	~9	0	9.6	0

Fig. 14. Absorption curves of myoglobin and derivatives in the visible and Soret regions. 0.1 mM metmyoglobin; 25 mM-Na₂S₂O₄; 40 mM-HCN for MetmbCN, 1 M-HCN for MbCN. Mb and MbCN measured in 0.1 M borax (pH 9.1-9.2); Metmb and MetmbCN measured in phosphate buffer pH 6.5. 1 cm. cells used for visible region, 1 mm. cells used for Soret region.

In Fig. 14 are collected the absorption curves within the range 650–400 $m\mu$. of Mb, MbCN, Metmb (acid form) and MetmbCN. A comparison of these curves with those of Fig. 9 brings forward in a striking manner the spectroscopic similarity of

Fig. 15. Absorption curves of myoglobin in the presence of different concentrations of HCN and at different pH values. 0.04 mm metmyoglobin with 25 mm- $\text{Na}_2\text{S}_2\text{O}_4$ in all cases except where stated. —, M-KCN; ---, 0.6M-KCN; —, 0.3M-KCN. pH adjusted with KH_2PO_4 or H_3PO_4 to give the following values for the numbered curves: i, 9.28; ii, 7.68; iii, 6.85; iv, 8.35; v, 5.70. ---, Metmb + $\text{Na}_2\text{S}_2\text{O}_4$ in 0.1 M borax; ---, Metmb + 0.3M-HCN (without $\text{Na}_2\text{S}_2\text{O}_4$). The last curve is independent of pH within the present range. 1 cm. stoppered cells.

different derivatives of peroxidase and metmyoglobin. The curves of MbCN and per^2CN are in fact superimposable almost completely within the range 700–450 $m\mu$., while those of the other three derivatives are very similar. In the Soret region there is close agreement in the positions of the band maxima of corresponding compounds but rather wide differences in the band heights.

In one respect, however, there is a striking contrast between the reactions of the two haemoproteins with cyanide. Although both per^2CN and MbCN are formed most readily at pH ~9 they behave differently as the pH is lowered. It has already been demonstrated (Figs. 10, 11) that acidification of per^2CN to pH 5 leads to the formation of per^3CN despite the presence of $\text{Na}_2\text{S}_2\text{O}_4$. A similar series of experiments with MbCN within the pH range 9.3–5.7 showed that free Mb and not MetmbCN was produced as the pH was lowered. These experiments are summarized in Fig. 15 where curves i–v, representing a series of solutions of progressively lower pH, are isobestic with the curve of Mb and not with that of MetmbCN. Owing to the difficulties of maintaining a high HCN concentration at the lower pH values it was necessary to decrease the cyanide concentration as the pH was lowered. However, the minimum cyanide concentration used (0.3M) would have ensured the complete formation of MetmbCN if any oxidation of myoglobin iron had occurred.

Photodissociation of MbCN. Attempts to detect an effect of Hg-435.8 $m\mu$. light on MbCN in the larger type of absorption cell revealed changes of extinction (at 566 $m\mu$.) that were too small to be considered significant. By using the microcells, however, we obtained small but definitely reversible effects of light. Solutions i, iii and v of Table 6 were subjected to alternate 1 min. periods of darkness and light, and the resulting changes of E^{566} are recorded, as A, B and C respectively, in Fig. 16. Whereas with per^2CN dissociation was more rapid than association, in the experiments on MbCN the reverse was observed, especially at the lowest HCN concentration (curve C). The association rates were in fact of the same order in both cases but there was a very marked difference in the dissociation rates. The comparative slowness of both processes in the case of MbCN allowed the reactions to be followed in the conventional manner and the more complex procedure adopted for per^2CN offered no advantage. Parallel measurements in absence of cyanide or $\text{Na}_2\text{S}_2\text{O}_4$ showed that the stray-light error was 0.002, i.e. less than 10% of the mean change of extinction. This correction was applied to the E values of solutions under illumination.

Photochemical efficiency of the dissociation of per^2CN

Although the main object of our spectrophotometric experiments was a quantitative study of the conditions governing the formation and dissociation of per^2CN , it became apparent that some of the results so obtained were of a type that could be used for an approximate estimate of the quantum efficiency of the reaction. The theoretical treatment

of such reactions was developed by Warburg & Negelein (1928*a, b*) and applied by them to the photodissociation of CO-pyridine haemochromogen and of HbCO (Warburg, Negelein & Christian, 1929). This work was carried out manometrically using a solution of pigment concentrated enough to take up a measurable volume of CO. At such concentrations the pigment absorbs the photodissociating light completely. The same mathematical treatment was extended by Bücher & Negelein (1942) to the study by an optical method of the photodissociation of HbCO and MbCO, and it is upon their procedure that our present calculations are based.

Fig. 16. Reversible photodissociation of MbCN during alternate 1 min. periods of light and darkness. Measurements made at the α -band maximum of MbCN. In all cases the solutions contained 0.04 mM metmyoglobin + 0.05 M $\text{Na}_2\text{S}_2\text{O}_4$. Cyanide-phosphate buffer was present as follows: A, M-KCN and 0.4 M KH_2PO_4 (pH 9.29); B, 0.635 M-KCN and 0.254 M KH_2PO_4 (pH 9.27); C, 0.273 M-KCN and 0.109 M KH_2PO_4 (pH 9.15). The micro-cells were used. The blank cell contained all ingredients except Mb. Dark (D) and light (L) periods indicated at top and bottom of the figure.

Bücher & Negelein have pointed out that precise results can be obtained by their method only if the solution of light-sensitive pigment is very dilute and contained in a long narrow optical cell. These conditions ensure: (a) that the effective concentration of the uncombined non-haematin constituent (e.g. CO), being present in large excess, remains virtually constant; and (b) that the decrease in

intensity of photodissociating light, on its passing through the narrow dimension of the solution, is not marked. Measurements of changes in absorption of the solution at other wavelengths are made in a direction at right angles to the photodissociating light path, and thus through the long dimension. In the experiments of Bücher & Negelein the solution transmitted more than 80 % of photodissociating light and the mean intensity within the solution could be readily calculated; in fact it was equal within experimental error to the mean of the incident and transmitted intensities. However, in our experiments, which utilized the 1×1 cm. cells, the solution transmitted less than 2 % of the Hg-435.8 μ . line, and estimates of the mean internal intensity were subject to considerable uncertainty. On the other hand, reason (a) (above) for using a long cell does not apply in our experiments since HCN concentration, unlike that of CO, is not restricted by solubility.

*Symbols used by Warburg & Negelein (1928*a, b*) and Bücher & Negelein (1942) in the calculation of quantum efficiency:*

- z , Photochemical dissociation constant (min.^{-1}).
- J_0 Intensity of photodissociating radiation falling on the solution (mole quanta $\text{min.}^{-1} \text{ cm.}^{-2}$).
- J Intensity of radiation transmitted by the solution.
- J_M Mean effective radiation intensity within the solution.
- β Absorption constant ($1/\text{cd} \ln J_0/J$).
- c Total haematin concentration (moles cm.^{-3}).
- d Optical depth for photodissociating radiation (cm.).
- q Area of cell illuminated by photodissociating radiation (cm.^2).
- ϕ Quantum efficiency (moles cyanide split off/mole quanta absorbed).
- ω_d Dissociation velocity constant of reaction 1 in darkness (min.^{-1}).
- ω_l Dissociation velocity constant of reaction 1 in light (min.^{-1}).
- ρ Association velocity constant of reaction 1 (min.^{-1}).

Thus the light-sensitive reaction of a ferro-haemoprotein (Fe) with either CO or HCN (X) can be represented as follows:

Additional symbols for the present calculations:

- T % transmission of the Hg-435.8 μ . line.
- $t_{\frac{1}{2}(\text{off})}$ } Times for 50 % completion of the dissociating and associating reactions (as in Fig. 13) (sec.).
- $t_{\frac{1}{2}(\text{on})}$ }

Bücher & Negelein (1942) express the rate of absorption of photodissociating light in terms of the difference between the incident and transmitted radiation

$$qJ_0(1 - e^{-\beta cd}) \text{ mole quanta min.}^{-1}. \quad (2)$$

When $J_0 - J$ is small compared with J_0 this expression reduces to $qJ_0\beta cd$ mole quanta min.⁻¹. This does not apply to our experiments where J_0 is no longer a good approximation for J . Since

$$e^{-\beta cd} = J/J_0 = T/100,$$

expression (2) becomes $qJ_{\infty}(1 - T/100)$ and moles per²CN split per minute is thus $\phi qJ_{\infty}(1 - T/100)$. Dividing this expression by the number of moles of pigment in the cell (qcd) gives the photochemical dissociation constant

$$z_J = \frac{\phi J_{\infty}(100 - T)}{100cd}. \quad (3)$$

As in Bücher & Negelein's experiments CO was present in excess over Mb the 'on' and 'off' reactions of MbCO can be considered as first-order reactions for which the velocity constants are $(\omega_d + \rho)$ and $(\omega_l + \rho)$ respectively. Since the curves of Fig. 13 approximate to those of first-order reactions we can write, for the reaction of per² with HCN,

$$\omega_d + \rho = \ln 2/t_{\frac{1}{2}(\text{on})} \quad (4)$$

$$\omega_l + \rho = \ln 2/t_{\frac{1}{2}(\text{off})}. \quad (5)$$

The determination of $t_{\frac{1}{2}(\text{on})}$ and $t_{\frac{1}{2}(\text{off})}$ enables the photochemical dissociation constant to be calculated from

$$z_J = (\omega_l + \rho) - (\omega_d + \rho). \quad (6)$$

The method of following the dissociation and association of per²CN is described above. By this procedure values of $t_{\frac{1}{2}(\text{on})}$ and $t_{\frac{1}{2}(\text{off})}$ were determined under the conditions given in Table 7. The concentrations of peroxidase and HCN were 0.0184 and 0.77 mM respectively, which assured a pseudo-unimolecular order for the association

reaction. Measurements of E^{565} on per² + HCN showed that the percentage per²CN formation was 35.6% in darkness and 17.8% in light. These figures indicate a smaller degree of reversibility than in the experiment of Fig. 13, but one which permits a more accurate estimate of J_{∞} . It would appear *a priori* that the narrow cells (Fig. 6) would be more favourable for this experiment, but they were in fact found unsuitable on account of the rapidity of the dissociation and also because $t_{\frac{1}{2}(\text{on})}$ was not reproducible. The latter uncertainty probably arose from the susceptibility of the factor a (below) to vary with extremely small variations in the position of the absorption cell in the monochromator beam. Such variations would have little effect when the larger cells, and a correspondingly wider monochromator beam, were used.

Substitution of the experimental values in Eqns. 4, 5 and 6 gives the result

$$z_J = 8.2 \text{ min.}^{-1}, \quad (7)$$

and ϕ can now be obtained from (3) and (7), provided that J_{∞} is known.

The relationship of J_{∞} to J_0 can be expressed in the form

$$J_{\infty} = abJ_0, \quad (8)$$

where a is the ratio of the mean intensity of Hg-blue light within the solution to the incident intensity and b is the fraction of the absorbed light which is absorbed by per²CN.

Evaluation of a . If light passes through a solution containing per² and per²CN, the mean effective intensity between two points distant x_1 and x_2 from the boundary where the light enters the solution will be

$$J_{\infty} = \frac{bJ_0}{x_2 - x_1} \int_{x_1}^{x_2} e^{-\beta cx} dx. \quad (9)$$

In the present experiments the exit beam from the monochromator was of approximately elliptical section where it entered the absorption cell (1 × 1 cm. horizontal dimensions) and was slightly divergent. The position of this beam within the cell

Table 7. Data for determination of quantum efficiency of photodissociation of per²CN

Half-reaction times for the association and dissociation in dark and light. 0.0184 mM peroxidase, 0.77 mM-HCN and ~25 mM-Na₂S₂O₄ dissolved in 0.1M borax (pH 9.1). Hg-435.8 mμ. intensity (J_0) = 8.37×10^{-7} mole quanta min.⁻¹ cm.⁻². Values of E^{540} before addition of Na₂S₂O₄ (per²CN) are used to calculate E^{565} values in last two lines. D, L, indicate equilibrium E values in darkness and in light. $t_{\frac{1}{2}(\text{on})}$ = time required in darkness, after prior illumination, for E^{565} to reach the mean of the D and L readings. $t_{\frac{1}{2}(\text{off})}$ = time required in light, after a period of darkness, for the mean E^{565} to be reached. Temp. = 22°.

Cell contents	E^{540}	E^{565}	% per ² CN	$t_{\frac{1}{2}}$ (sec.)	Mean
per ² + HCN	D, 0.194 L, 0.194	— —	— —	— —	— —
per ² + HCN + Na ₂ S ₂ O ₄	—	D, 0.246 L, 0.220	35.6 17.8	On: 6.68, 6.31, 6.44, 6.60, 6.59 Off: 2.87, 2.83, 2.72, 2.98	6.52 2.85
100 % per ²	—	0.340	Calculated	—	
100 % per ² CN	—	0.198			

and the mean size of its cross-section are shown as an ellipse in Fig. 17. In order to calculate the mean intensity of Hg-blue light within this beam a rectangle of equal area was constructed (Fig. 17) and it was assumed that the mean intensity within such a rectangular beam would be equal to that within the elliptical beam. The values of x_1 and x_2 (Fig. 17) were found to be 0.21 and 0.74 cm. respectively, and therefore (9) becomes

$$J_{\Sigma} = \frac{bJ_0}{0.53} \int_{0.21}^{0.74} e^{-\beta x} dx \\ = 0.51 bJ_0 \text{ (i.e. } a = 0.51).$$

Fig. 17. Representation to scale of the position of the monochromator beam within a 1 x 1 cm. absorption cell as it would appear if viewed from the monochromator exit slit. The cross-section of the beam is indicated by the ellipse upon which is constructed a rectangle of equal height and area, and situated symmetrically about OA. The broken curve indicates logarithmic decline of Hg-blue light as it passes through the solution; the intensities being represented by the height of this curve above the dotted base-line. Thus the factor a is the ratio of the mean height of the shaded area to J_0 .

Evaluation of b . From the mean value of E_{565}^{565} during the period $0-t_{1/2(\text{cm})}$ (0.240) it can be calculated that the mean mole fraction of per^2CN during the period is 0.30. Using the data on extinction at 435.8 m μ . (Fig. 9) the fraction of absorbed Hg-blue light absorbed by per^2CN (b of Eqn. 8) can be shown to be 0.436.

Measurement of Hg-blue light-intensity falling on a spectrophotometer cell (type iii, 1 cm. square cross-section) was carried out by means of the potassium ferrioxalate actinometer of Parker (1953). 3 ml. of 0.17 M ferrioxalate in 0.1 N- H_2SO_4 was placed in the cell, thus exposing 3 cm.² to the blue light. The cell contents were stirred by means of a fine stream of N_2 and irradiated for exactly 1 min. The ferrous ion was estimated by Parker's procedure except

that 0.01 M $\alpha\alpha'$ -dipyridyl replaced the 0.1% solution of 1:10-phenanthroline. Under the above conditions the quantum efficiency of this reaction, according to Parker, is approximately 0.89.

A determination made immediately after the experiment described above showed the formation of 2.24×10^{-6} g.-atoms Fe^{2+} . Thus the light intensity, J_0 , was

$$(2.24 \times 10^{-6}) / (0.89 \times 3) = 8.37 \times 10^{-7} \text{ mole} \\ \text{quanta min.}^{-1} \text{ cm.}^{-2}.$$

Eqn. 8 now becomes

$$J_{\Sigma} = 0.222 J_0 = 1.86 \times 10^{-7} \text{ mole quanta} \\ \text{min.}^{-1} \text{ cm.}^{-2},$$

and introduction of the values of J_{Σ} , T (1.7), c (1.84×10^{-8}), d (1) and z , [Eqn. 7] in Eqn. 3 gives $\phi = 0.83$. This figure must be regarded as approximate because (i) there is considerable uncertainty in the estimation of a , (ii) the intensity of the Hg lamp tended to fluctuate, and (iii) the response of the Beckman spectrophotometer is scarcely rapid enough for accurate measurement of the low values of $t_{1/2}$. It can, however, be concluded that the quantum efficiency approximates to unity.

DISCUSSION

Cyanide compounds of peroxidase and myoglobin

It has previously been established that cyanide combines reversibly with ferriperoxidase (per^3) forming a well-defined per^3CN compound, the absorption spectrum of which shows a close similarity to those of cyanide compounds of methaemoglobin (Methb) and catalase (Keilin & Mann, 1937; Keilin & Hartree, 1951). The formation of per^3CN takes place over a wide range of pH (4.6–10.3) and the compound is very stable. Its dissociation constant estimated approximately in the course of this work is 2×10^{-6} M which is not far from the value of 4×10^{-6} M, previously recorded by Chance (1943). The affinity for cyanide is thus very high.

The reaction of cyanide with ferriperoxidase (per^3) and the formation of per^2CN are described for the first time in this paper. The complete formation of this compound requires a higher concentration of HCN and takes place within a much more limited range of pH 8.5–9.5. The absorption spectrum of per^2CN is of the haemochromogen type showing three absorption bands: α -565.5 m μ . ($\epsilon_{\text{mM}} = 18.5$); β -535 m μ . ($\epsilon_{\text{mM}} = 13.6$); γ -432.5 m μ . ($\epsilon_{\text{mM}} = 135$). This absorption spectrum differs, however, from that of a typical protohaemochromogen in having its α - and β -bands about 7 m μ . nearer the red end of the spectrum and in a somewhat lower extinction of the α -band. Thus the

α -bands of protohaemochromogens formed by different amino acids lie at 556–558 $m\mu$., while $\epsilon_{\max}$ varies between 21 and 30 (J. Keilin, 1950). The dissociation constant of per^2CN is about $1.3 \times 10^{-3} \text{M}$, which shows that the affinity of cyanide for per^2 is about 1000 times lower than for per^3 . Thus the pH ranges of the reactions of per^2 and per^3 with cyanide and their relative affinities for cyanide clearly indicate that the reversible inhibition of the catalytic activity of this enzyme by cyanide, which was already known to Linossier (1898), is due entirely to the formation of per^3CN .

The formation of per^2CN at pH 9 can be easily observed following the addition of HCN to per^2 or of $\text{Na}_2\text{S}_2\text{O}_4$ to per^3CN . On making the solution more acid, per^2CN is formed incompletely, and below pH 7 the partly formed per^2CN is in equilibrium, not with free per^2 but with per^3CN . At pH 5–6 even in presence of $\text{Na}_2\text{S}_2\text{O}_4$ only per^3CN can be seen spectroscopically. Thus, $\text{Na}_2\text{S}_2\text{O}_4$, which at pH 5–6 still easily reduces ferric haematin compounds such as per^3 , Methb, parahaematin and haematin to the corresponding ferrous derivatives, fails to reduce per^3CN to per^2CN . In this respect the tervalent iron of per^3CN at pH 5–6 resembles that of catalase which, while the enzyme is still in the native state, cannot be reduced by $\text{Na}_2\text{S}_2\text{O}_4$. Cyanide, therefore, appears to lower markedly the oxidoreduction potential of peroxidase, the E'_0 value of which at pH 7.30 and 30° in absence of cyanide is already as low as -0.27V (Harbury, 1953).

Like other haem compounds per^2 combines reversibly with CO, for which it has much greater affinity than for cyanide, the value of the partition or equilibrium constant

$$K = ([\text{per}^2\text{CO}] [\text{HCN}]) / ([\text{per}^2\text{CN}] [\text{CO}])$$

being approximately 2500.

The easiest way of observing the formation of HbCN or MbCN is by reduction with $\text{Na}_2\text{S}_2\text{O}_4$ of MethbCN or MetmbCN in the presence of a large excess of HCN. As stated above, only MbCN is sufficiently stable for precise spectrophotometric measurements. The optimum pH for the formation of MbCN (9.3) is close to that for per^2CN while an even higher cyanide concentration (1M) is required. The absorption spectra of HbCN, MbCN and per^2CN are indistinguishable by visual spectroscopy while precise measurement of light absorption of MbCN and per^2CN in the visible and Soret regions gave curves which are virtually superposable. This close similarity appears to be unique among native haemoprotein derivatives. Thus from among the derivatives of haemoglobin, peroxidase, and catalase it is possible to select groups of homologous compounds (e.g. *a*, Hb and per^2 ; *b*, Methb, per^3 and catalase; *c*, MethbCN,

per^3CN and cyan-catalase). Although the members of each group show a degree of similarity of spectral pattern (Keilin & Hartree, 1951; Figs. 9 and 14 of the present paper), they can nevertheless be distinguished spectroscopically as long as the protein is in the native state.

HbCN, MbCN and per^2CN can be considered as hybrid haemochromogens analogous to nicotine-cyanide haemochromogen (Hill, 1929) or globin-cyanide haemochromogen (Anson & Mirsky, 1930). The shift of the band maxima towards the red may be related to the fact that whereas in amino acid or denatured protein haemochromogens it is the nitrogen atoms that are linked to the iron atom through the 5th and 6th co-ordination valencies, the cyanide group in a hybrid haemochromogen is linked to iron via the carbon atom (J. Keilin, 1949).

Although the experiments so far carried out do not permit a clear decision to be made on the question whether HCN or CN^- reacts with per^2 to form per^2CN , the right-hand portion of the curve in Fig. 11 (pH 9–11) is consistent with a reaction of per^2 with the free acid.

Cyanide and carbylamine derivatives: their reactions with CO

Cyanide is known to combine with the bivalent and tervalent iron of haem and haematin respectively, both when they are free and when, as prosthetic groups, they form part of haemoglobin and methaemoglobin. Haem may form with cyanide two distinct compounds: monocyano- and dicyano-haem, showing absorption spectra of haemochromogen and dihydroxyhaem types respectively. Moreover, dicyanohaem, on reacting with CO, forms CO-cyanhaem in which the bivalent iron of free haem is linked simultaneously to two respiratory inhibitors (CO and HCN) through co-ordination valencies 5 and 6 (J. Keilin, 1949, 1955). However, when haem forms part of a haemoprotein molecule one of these valencies unites the iron to the protein, while the other links it to a molecule of water, which can be replaced either by CO or by cyanide, both competing for the bivalent iron.

Carbylamines, e.g. methyl isocyanide ($\text{CH}_3.\text{NC}$), also combine with haem and haemoglobin (Warburg *et al.* 1929). With proto- and uro-haem carbylamine forms two compounds: mono- and dicarbylaminehaem, the absorption spectra of which are very similar to those of the corresponding cyanhaem compounds and, like dicyanohaem, dicarbylaminehaem reacts with CO to give CO- $\text{CH}_3.\text{NC}$ haem (J. Keilin, 1949). However, with haemoglobin, carbylamine forms only one compound and, contrary to the view developed by Warburg *et al.* (1929), this compound contains only one molecule of $\text{CH}_3.\text{NC}/\text{Fe}$ atom and this can be replaced by a molecule of O_2 or CO. The fact that

$\text{CH}_3\text{.NC}$ competes with CO for the iron explains the success of the quantitative manometric study of photodissociation of HbCO in presence of $\text{CH}_3\text{.NC}$ (Warburg *et al.* 1929). The absorption spectra of $\text{per}^2\text{CH}_3\text{.NC}$ and $\text{MbCH}_3\text{.NC}$ in the visible region (Fig. 18) are of the same pattern as the corresponding cyanide derivatives, except that the extinction coefficients of both the α - and β -bands are much lower.

Fig. 18. Absorption curves of ferroperoxidase and haemoglobin and their methylcarbamylamine derivatives. 0.04 mM haemoprotein (per^2 or oxyhaemoglobin), 25 mM- $\text{Na}_2\text{S}_2\text{O}_4$, 0.1 M borax. 80 mM carbamylamine for $\text{per}^2\text{CH}_3\text{.NC}$ curve; 1.1 mM carbamylamine for $\text{HbCH}_3\text{.NC}$ curve (these concentrations are minimal for complete compound formation in each case). 1 cm. stoppered cells; pH 8.8–9.0.

Photodissociation of CO compounds and calculation of quantum efficiency

In 1896 Haldane & Smith discovered that HbCO reversibly dissociates in light. More than 30 years later this important discovery was followed by extensive quantitative studies carried out by Warburg and his co-workers on the effect of light on CO derivatives of haem and of haemoproteins. In these studies calculation of the quantum efficiency of the photodissociation was based upon a theoretical treatment by Warburg (1928) and applied by his school to manometric studies of the photodissociation of CO-pyridine haemochromogen (Warburg & Negelein, 1928*a, b*) and of HbCO in presence of carbamylamine (Warburg *et al.* 1929). The

same treatment was later extended to an optical study of photodissociation of HbCO and MbCO in presence of O_2 and also of CO-pyridine haemochromogen (Bücher & Negelein, 1942). Not all ferrohaemoproteins combine with CO, and of those that do combine not all can be seen to dissociate in light either by spectroscopic observation or by spectrophotometric examination as described for per^2CO (p.162). Photodissociation is clearly shown, however, when CO competes for co-ordination valency 6 of the iron atom not with a molecule of water nor a hydroxyl group but with such other ligands as pyridine in pyridine haemochromogen, a haem-linked group of protein in certain protein haemochromogens (e.g. the CO compound of alkaline ferrocyclochrome *c*), O_2 in oxyhaemoglobin or $\text{CH}_3\text{.NC}$ in $\text{HbCH}_3\text{.NC}$. In fact, the photodissociation of CO compounds of free haem and of Hb (both in the presence of $\text{Na}_2\text{S}_2\text{O}_4$), when CO is competing with water, is not easily observed.

Since O_2 in oxyhaemoglobin is replaced by the same amount of CO, the photodissociation of HbCO cannot be followed manometrically in presence of O_2 , but can be easily followed under anaerobic conditions in the presence of carbamylamine, the method which was successfully applied for this purpose by Warburg *et al.* (1929). The optical method introduced by Bücher & Negelein (1942) is not only free from this limitation but also requires only very dilute solutions of the pigment. These workers corroborated with their optical method the manometric results of Warburg *et al.* They give the following values for (mole quanta absorbed)/(moles CO split off), which is thus the reciprocal of the quantum efficiency (see p.165):

CO-pyridine haemochromogen	0.93
CO-myoglobin	1.1
CO-haemoglobin, pH 7.6	3.38
CO-haemoglobin, pH 9.1	4.05
CO-haemoglobin, pH 7.6 in the presence of 2.6 M-NaCl	2.18

The low efficiency in the case of HbCO , where the absorption of 4 quanta liberated only one molecule of CO, has so far received no satisfactory explanation.

Photodissociation of per^2CN

The light sensitivity of per^2CN and of MbCN which is described in this paper appears to be unique among cyanide derivatives and finds a parallel only among the CO compounds of haem and its derivatives. The marked light sensitivity of per^2CN , in spite of the fact that cyanide replaces a molecule of water in this complex, may partly arise through the low affinity of cyanide for per^2 .

The reversible photodissociation of per^2CN and MbCN bears no resemblance to the irreversible

photodecomposition of certain complex cyanides such as ferricyanides and molybdicyanides (Bucknall & Wardlaw, 1927). In this connexion it may be interesting to recall that under the name of photo-methaemoglobin Bock (1895) described a derivative he obtained by exposing Methb to strong light. This derivative was soon recognized by Haldane (1900) as a compound of Methb with cyanide which was produced by an irreversible photodecomposition of ferricyanide present in the Methb solution. This explanation was later confirmed by Zeynek (1901).

It is not surprising that the discovery of the reversible photodissociation of per^2CN was made during observations with the microspectroscope, since with this technique it is possible to detect rapid spectral changes from the time that the reactants are mixed. Furthermore, the customary use of very strong illumination made the changes both clearly marked and rapid; in fact it is very doubtful if the photochemical effect would have been detected had the experimental methods been limited to measurements in a spectrophotometer. Once the phenomenon had been established a quantitative study could be carried out in a Beckman quartz spectrophotometer with a modified cell-carrier which enabled both cells (solution and solvent) to be illuminated from the side with light within the spectral region of the intense γ -band. It is fortunate that the readily isolated and intense 435.8 m μ . Hg line is close to the γ maxima of per^2CN and MbCN lying at 432.5 and 434 m μ . respectively. By suitable selection of complementary light-filters it was found possible to measure changes of absorption at 565 m μ . (α -max.) without the Hg-blue side-illumination affecting the photocell of the spectrophotometer.

Although the photodissociation of MbCN is not as marked as that of per^2CN , a small but definite reversible effect of light could be recorded (Fig. 16).

No light sensitivity has so far been detected in any other cyan-haem compound or in $\text{per}^2\text{CH}_3\text{NC}$ and MbCH_3NC . This does not exclude the possibility that under other experimental conditions further examples of light-sensitive haematin compounds may be found. On the other hand, per^2CO , like many other CO haemoproteins and their haem derivatives, was found to be light-sensitive (Table 4). This explains the effect of light on the CO inhibition of the oxidation of dihydroxyfumaric acid catalysed by peroxidase (Swedin & Theorell, 1940).

Light efficiency in photodissociation of per^2CN

Although the apparatus developed for the study of photodissociation is not suited for the measurement of quantum efficiency, nevertheless it was considered that the experimental results on rates of association and dissociation of per^2CN , in

darkness and in light respectively, would enable an approximate figure for the quantum efficiency to be obtained. The chief disadvantage of the present method, and the one which introduces the greatest uncertainty, is that the Hg-blue light is completely absorbed by the experimental solution. Under such conditions precise calculations of the mean light intensity within the solution are difficult to make.

In calculating the quantum efficiency we have followed the principles laid down by Warburg (1928) and further developed by Bücher & Negelein (1942). Data on the intensity of Hg-blue light falling on the spectrophotometer cell were obtained by means of the ferrioxalate actinometer of Parker (1953) which, though simpler, is rather less accurate than the direct estimation with a bolometer: the method used by previous workers. Our data give a figure of 0.83 for the quantum efficiency in photodissociation of per^2CN , but in view of the uncertainties in our calculation we can only conclude that the efficiency is of the order of unity.

SUMMARY

1. Ferriperoxidase (per^3) combines reversibly with cyanide over a wide range of pH (4.6–10.3) forming a stable ferriperoxidase-cyanide complex (per^3CN) with an absorption spectrum very similar to that of metmyoglobin cyanide (MetmbCN) or catalase cyanide.

2. Ferropoxidase (per^2) also combines reversibly with cyanide but within a much more limited range of pH (8.5–9.5) forming the less stable per^2CN which can be obtained either by addition of HCN to per^2 or of $\text{Na}_2\text{S}_2\text{O}_4$ to per^3CN .

3. The affinity of per^2 for cyanide is about 1000 times lower than that of per^3 , the dissociation constants at pH 9 being $1.3 \times 10^{-3}\text{M}$ for per^2CN and $2 \times 10^{-6}\text{M}$ for per^3CN .

4. The absorption spectrum of per^2CN is identical with that of HbCN and MbCN. It is of the haemochromogen type, the positions and intensities of bands being: α -565.5 m μ . ($\epsilon_{\text{max}} = 18.5$); β -535 m μ . ($\epsilon_{\text{max}} = 13$); γ -432.5 m μ . ($\epsilon_{\text{max}} = 135$).

5. On acidifying a solution of per^2CN the above bands gradually disappear and are replaced, not by those of per^2 , but by the absorption bands of per^3CN , the change being complete at pH < 6.

6. At pH 5.5–6.0 per^2CN does not undergo reduction even in the presence of $\text{Na}_2\text{S}_2\text{O}_4$; in this respect the compound resembles catalase.

7. Per^2 combines reversibly with CO, forming a light-sensitive per^2CO compound. The affinity of per^2 for CO is much greater than for cyanide, the partition constant

$$K = \frac{[\text{per}^2\text{CO}][\text{HCN}]}{[\text{per}^2\text{CN}][\text{CO}]}$$

being about 2500.

8. Per² combines also with methylcarbylamine, forming a compound very similar to the carbylamine compounds of Hb and Mb.

9. Per²CN undergoes a reversible photodissociation which can easily be followed by direct micro-spectroscopic observation. The light sensitivity of this compound is of special interest considering that hitherto the only compounds of haems known to be light-sensitive were their CO compounds.

10. A method is described for quantitative spectrophotometric study of the kinetics of reversible dissociation of per²CN. The photodissociation was effected by means of Hg-blue light the wavelength of which (435.8 mμ.) lies close to the intense γ-band of per²CN.

11. Examination of MbCN by the same method revealed a less marked but nevertheless distinct light sensitivity.

12. An attempt to calculate, from the spectrophotometric experiments, the quantum efficiency in photodissociation of per²CN gave an approximate value of $\phi = 0.8$ (moles per²CN dissociated per mole light quantum absorbed). In view of the uncertainties in the calculation this result indicates only that the efficiency is of the order of 1.

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Transfructosidase from *Agave vera cruz* Mill

By I. S. BHATIA, M. N. SATYANARAYANA AND M. SRINIVASAN

Division of Quality Control, Central Food Technological Research Institute, Mysore

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The nature and distribution of carbohydrates in *Agave vera cruz* have been reported on by Srinivasan & Bhatia (1953, 1954a). The isolation and properties of an enzyme present in the stem of this plant, capable of building glucofructosans from sucrose, are described in this paper. So far, the only other plant sources shown to contain such an enzyme are Jerusalem artichoke (Edelman & Bacon, 1950, 1951; Dedonder, 1952) and *Polianthes tuberosa* (Bhatia & Srinivasan, 1954), the latter yet another glucofructosan-bearing material (Srinivasan & Bhatia, 1954b).

EXPERIMENTAL

Crude extract. The outer skin was scraped from an agave stem (see Srinivasan & Bhatia, 1953) and the bottom and top ends (3 in. each) were discarded. The remaining stem was then sliced. Some slices representing different sections of the stem were used for examination of microflora. The rest of the slices were treated with CHCl₃-water, grated and pressed through cloth. The pressed juice was centrifuged (2000 rev./min., 20 min.) and filtered. The filtrate, hereafter referred to as the crude extract, was stored over CHCl₃ in the cold room (3°). The slices, gratings and the crude extract were found to be free from microflora, when tested as follows.