at 280 and 294·4 mμ., the total protein content can be calculated from the expression

Total protein (g./100 ml.) = 9·76 (2·2$E_{294.4}$ − E_{280}).

Estimation of serum protein concentration from serum specific gravity. The relationship between the serum protein concentration (P, in g./100 ml.) and the serum specific gravity (G) is usually expressed in the form

$$P = a(G − b)$$

where a and b are constants.

Sera from 320 cattle have been examined, the serum protein values varying from 4·5 to 11·0 g./100 ml. and the specific gravities from 1·0200 to 1·0405. For the specific gravity range 1·0220 to 1·0365 (corresponding to a serum protein range of from 5·5 to 9·6 g.) the equation relating these quantities, obtained using values for 312 sera, was

$$P = 362·0 \ (G − 1·0020)$$

($a = 362·02 \pm 7·668$, $b = 1·00197 \pm 0·000202$). Beyond these limits the relationship was no longer linear.

DISCUSSION

The value for the constant (1·0020) obtained for cattle in the equation relating serum protein con-centrations and serum specific gravity is low com-pared with that found for the plasma of other species (Van Slyke, Hiller, Phillips, Hamilton, Dole, Archibald & Eder, 1950). The value for this constant should agree approximately with the specific gravity of a solution of the serum crystalloids. The sera were obtained from adult zebu cattle which were undoubtedly existing on a low plane of mineral nutrition, and it is possible that the low value of b reflects a lowered serum content of mineral salts.

SUMMARY

1. The tyrosine content of mixed bovine serum proteins has been determined. 1 mg. of tyrosine is contained in 19·78 mg. of total protein (tyrosine determined using the Folin & Ciocalteu reagent) or 19·68 mg. of total protein (tyrosine determined spectrophotometrically).

2. A linear relationship exists between the serum protein concentration and the serum specific gravity, provided that the latter falls within the range 1·0220 to 1·0365. Within these limits

$$P = 362·0 \ (G − 1·0020).$$

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Reaction Between Hydrocyanic Acid, Cyanide Ion and Ferricytochrome *c*

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Potter & Lockhart (1939) reported that the enzymic reduction of cytochrome *c* by either the dihydro-cozymase-diaphorase system or the succinic de-hydrogenase system was blocked by 0·01 M-cyanide. In later communications (Lockhart & Potter, 1941;

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Potter, 1941), it was concluded that cytochrome *c* was involved in a reaction with cyanide. The evidence presented to support this conclusion can be summarized as follows. (1) By incubation of cyto-chrome *c* with cyanide, the enzymic reduction of the former was partly or completely prevented, de-pending on the cyanide concentration, and on the time and pH of incubation. The enzyme systems

which are responsible for the reduction of cyto-chrome *c* were not affected. (2) Incubation with cyanide also shifted the absorption maximum of ferricytochrome *c* at 530 mμ. about 5 mμ. toward the red.

This conclusion was later confirmed by Horecker & Kornberg (1946), who studied this reaction in con-siderable detail and suggested that complex forma-tion involved only the free cyanide ion. These workers demonstrated the reversibility of the re-action and determined the formation velocity con-stant and equilibrium constant from which they calculated the dissociation velocity constant. They also obtained values for the heat of dissociation and the energy of activation for the formation of the complex. The absorption spectrum of the ferricyto-chrome *c*-cyanide complex, obtained by Potter in the green region, was extended by these workers to the red. They found that the feeble bands of ferri-cytochrome *c* at 655 and 695 mμ. disappeared when it was converted to the complex.

In spite of the conclusive evidence put forward by these workers, the fact that cytochrome *c* forms a reversible complex with cyanide does not seem to have been generally accepted (see, for instance, Theorell, 1947; Wyman, 1948). In the present in-vestigation, some of the results of Potter and of Horecker & Kornberg have been confirmed.

The formation velocity constant and the equi-librium constant of the reaction have been deter-mined at different temperatures and over a wider pH range than hitherto investigated. Evidence is presented to show that ferricytochrome *c* is capable of reacting with both hydrocyanic acid and free cyanide ion; the former reaction predominates in acid solutions and the latter in neutral and slightly alkaline solutions. From the kinetic and equi-librium data forward and back velocity constants and the two equilibrium constants are calculated, together with heats and entropies of activation and the overall change in heat content and entropy associated with the formation of the complex. Where comparison is possible, the numerical values of those quantities differ from those reported by Horecker & Kornberg as would be expected because at the pH of their experiments, i.e. 7·4, the reactions with both hydrocyanic acid and cyanide ion are important, whereas they analysed their data in terms of a reaction with cyanide ion alone.

Experiments are also presented showing that the velocity constant for the formation of the complex decreases above pH 9·2, which may be attributed to an ionization in the ferricytochrome *c* molecule such that the formation of the complex occurs pre-dominantly by reaction of the acid form.

It has been shown previously, however, that endo-genous cytochrome *c*, i.e. cytochrome *c* as present in living cells and in certain colloidal enzyme prepara-tions, does not react with cyanide (Tsou, 1951*b*). The present paper deals exclusively with exo-genous cytochrome *c*.

EXPERIMENTAL

Cytochrome c of iron content 0·34 % was prepared by the method of Keilin & Hartree (1945).

Heart-muscle preparation containing the enzymes re-sponsible for the reduction of cytochrome *c* by succinate was prepared according to the Keilin & Hartree method as previously described (Tsou, 1951).

Cyanide. Neutral solutions were prepared each week and the cyanide concentration was determined by titration against standard silver nitrate solution immediately before use.

Following Latimer (1938), we have taken the results of Harman & Worley (1924) on the hydrolysis of KCN as the most reliable data for the ionization constant of HCN, K_a. From their values of the hydrolysis constant at various temperatures and the values for K_w, the ionic product of water, at temperatures 15–40° given by Harned & Owen (1943), we have calculated the values of K_a appropriate to the temperatures of our experiments. These are given later in Table 3. We have made no correction for ionic strength, partly because Harman & Worley's experiments were carried out at an ionic strength comparable to that em-ployed in the present investigation. However, in calcu-lating K_a from the hydrolysis constant a value of K_w corrected for ionic strength should strictly be used. But in view of the uncertainty in the hydrolysis data shown by the different results obtained by previous workers, e.g. Madsen (1901) gives a value for K_a of $4·7 \times 10^{-10}$ at 18°, whereas the value calculated from Harman & Worley's data is about $2·5 \times 10^{-10}$, and considering that the correction to K_w would only be of the order of 15 %, we have chosen to neglect it. It must be borne in mind that values of velocity constants and equilibrium constants obtained later are subject to correction if a better value of K_a becomes available.

Buffers. Below pH 8·0, 0·075 M-phosphate buffers were used; and in the pH range 8·0–10·0, 0·075 M-borate buffers. pH measurements were made with a glass electrode and a Cambridge pH meter.

Light absorption. This was measured with a Beckman photoelectric spectrophotometer.

Estimation of reaction rate. The cytochrome *c* solution used was first acidified to about pH 3–4 and aerated to ensure complete oxidation. It was then titrated back to neutrality. This solution (1 ml.) was mixed with 5 ml. of 0·15 M-buffer of the required pH, the correct amount of neutralized cyanide, and water to make a total volume of 10 ml. (solution *A*). The pH of the solution was measured immediately at the end of the experiment at each temperature with a glass electrode assembly. Solution *B* was made up from 1 ml. of heart-muscle preparation, 5 ml. of 0·4 M-succinate and 0·25 M-phosphate buffer, pH 7·4, to a total volume of 50 ml. Periodically, 1 ml. portions of solution *A* were pipetted into 2 ml. portions of solution *B*. The optical density at 550 mμ. of such a mixture was read against a blank in a Beckman spectrophotometer until a maximum value was reached (usually after 10–30 sec.). The blank contained the same concentrations of all the reagents except cytochrome *c*. At this stage, the uncombined cytochrome *c* was fully reduced.

The molar concentration of free cytochrome c, $[c]$, at any time t can be calculated from the following equation

$$[c] = \frac{D_t - D_\infty}{1 \cdot 88 \times 10^4} \times 3, \qquad \text{(i)}$$

where D_t is the steady density of the mixture prepared at time t; D_∞ is the density when all the cytochrome is converted to the cyanide complex prior to mixing with the heart-muscle preparation. This figure can also be obtained by
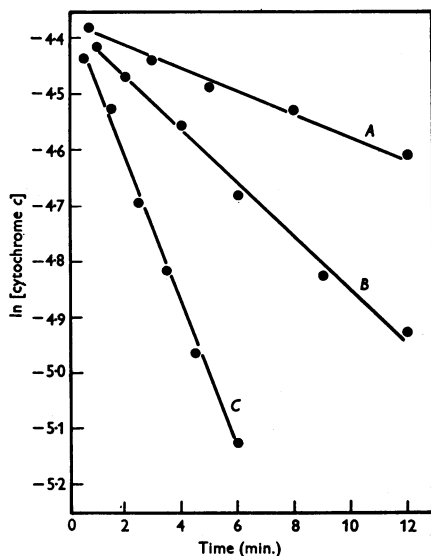

Fig. 1. Reaction of ferricytochrome c with cyanide. Plot of logarithm of (free cytochrome c concentration) against time, showing that the reaction follows the first-order equation

$$-\frac{2 \cdot 3 \; d \ln [c]}{dt} = k_{obs.} [KCN].$$

Temp. $= 24 \cdot 6^\circ$, pH $= 7 \cdot 4$, and total cyanide concentrations were $4 \cdot 2$ (A), $10 \cdot 3$ (B), and $25 \cdot 8$ (C) mM respectively.

multiplying the total concentration of cytochrome c by $0 \cdot 92 \times 10^4$ which is the molecular extinction coefficient of the complex at 450 mμ. (Tsou, 1952); and $1 \cdot 88 \times 10^4$ is the difference between the molecular extinction coefficient of ferrocytochrome c (Theorell & Åkeson, 1941 a) and that of the complex at 550 mμ. When KCN is present in excess, the reaction is first order and the velocity constant is calculated from

$$k_{obs.} = -\frac{2 \cdot 3}{[KCN]} \times \frac{d \ln [c]}{dt}, \qquad \text{(ii)}$$

where [KCN] is the concentration of total cyanide and d log $[c]/dt$ was obtained graphically by plotting $\ln [c]$ against time (Fig. 1).

Estimation of equilibrium constant. The same method is also available for estimation of the equilibrium constant of the reaction between cytochrome c and cyanide, except that lower cyanide concentrations and longer incubation times have to be used. Precautions to prevent loss of HCN vapour were necessary (Horecker & Kornberg, 1946).

RESULTS

Order of reaction. In presence of excess cyanide, the reaction followed the first-order Eqn. ii, as is shown in Fig. 1. These first-order constants, $k_{obs.}$, were found to depend on the hydrogen-ion concentration, as shown in Fig. 2, where the values obtained at $11 \cdot 7$ and $24 \cdot 6^\circ$ are plotted as a function of pH over the range $6 \cdot 0$–$10 \cdot 0$. Similar results were obtained at $18 \cdot 2$ and $30 \cdot 4^\circ$ for the pH range $6 \cdot 0$–$8 \cdot 0$.
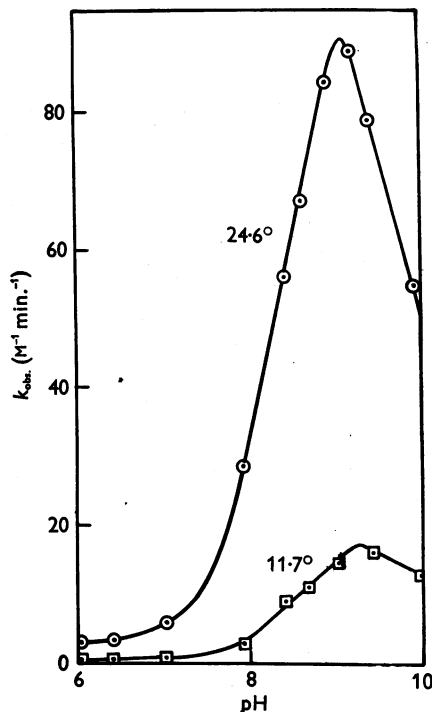

Fig. 2. Variation of the observed velocity constant for the formation of the ferricytochrome c-cyanide complex with the hydrogen-ion concentration, at $11 \cdot 7$ and $24 \cdot 6^\circ$, in $0 \cdot 075$ M-phosphate and borate buffers.

Equilibrium measurements. Using the method described above, the equilibrium concentration of the ferricytochrome c-cyanide complex in solutions containing ferricytochrome c and potassium cyanide was measured at several potassium cyanide concentrations over the pH range $5 \cdot 8$–$7 \cdot 5$. From the results an equilibrium constant K_{KCN} was calculated for the overall reaction from the equation

$$K_{KCN} = \frac{[\text{cyt. } c\text{-CN}]}{[\text{cyt. } c] \times [\text{KCN}]}. \qquad \text{(iii)}$$

It will be shown below that this overall equilibrium constant is compounded of two equilibrium constants appropriate to the two paths the reaction can take, one involving the un-ionized HCN molecule

and the other involving the free cyanide ion. By expressing the experimental results in this form, i.e. Eqn. iii, they are more amenable to analysis. Values of this equilibrium constant over the pH range 5·8–7·5, at 13·8 and 24·6°, are recorded in Tables 1 and 2.

Table 1. *Equilibrium constant data at 13·8° for the formation of the ferricytochrome c-cyanide complex calculated from Eqn. iii at different hydrogen-ion concentrations*

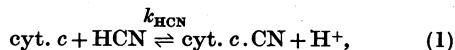| pH	KCN (mM)	$K_{KCN}(\times 10^{-3} M)$	Mean $K_{KCN}(\times 10^{-3} M)$
5·86	2·9	0·194	0·200
	0·97	0·206	
6·30	1·95	0·526	0·513
	0·65	0·500	
6·74	0·97	1·61	1·48
	0·32	1·35	
6·98	0·65	2·47	2·38
	0·22	2·29	
7·41	0·44	5·55	5·30
	0·22	5·05	

Table 2. *Equilibrium constant data at 24·6°*

(K_{KCN} values are calculated from Eqn. iii.)

pH	KCN (mM)	$K_{KCN}(\times 10^{-3} M)$	Mean $K_{KCN}(\times 10^{-3} M)$
5·83	2·9	0·319	0·323
	0·97	0·327	
6·25	1·95	0·952	0·889
	0·65	0·826	
6·72	0·65	2·14	2·15
	0·22	2·16	
6·98	0·44	4·42	4·36
	0·22	4·30	
7·46	0·33	14·3	13·0
	0·16	11·6	

ANALYSIS OF THE RESULTS

An examination of the hydrogen-ion dependence of the bimolecular velocity constant for the formation of the complex given in Fig. 2 shows that at high hydrogen-ion concentrations, up to about pH 6·0, the velocity constant is independent of pH and has a low value, but that as the solution is made more alkaline, pH 7·0–9·0, the velocity constant increases rapidly. This suggests that in the pH range 6·0–9·0 we are dealing with two reactions giving the complex, the first with HCN and the second with CN⁻:

$$\text{cyt. } c + \text{HCN} \xrightleftharpoons{k_{HCN}} \text{cyt. } c.\text{CN} + \text{H}^+, \qquad (1)$$

$$\text{cyt. } c + \text{CN}^- \xrightleftharpoons{k_{CN^-}} \text{cyt. } c.\text{CN}. \qquad (2)$$

Representing the individual velocity constants for these two reactions by k_{HCN} and k_{CN^-}, it can be

shown that the observed bimolecular velocity constant, $k_{obs.}$, should be given by

$$k_{obs.} = \frac{k_{HCN} \cdot [\text{H}^+]}{[\text{H}^+] + K_a} + \frac{k_{CN^-} \cdot K_a}{[\text{H}^+] + K_a}, \qquad (iv)$$

since the fraction of un-ionized HCN and the cyanide ion at any hydrogen-ion concentration are given by $\frac{[\text{H}^+]}{[\text{H}^+] + K_a}$ and $\frac{K_a}{[\text{H}^+] + K_a}$, respectively, where K_a is the ionization constant of HCN. Thus, to test the mechanism given by reactions 1 and 2 above, $k_{obs.}([\text{H}^+] + K_a)$ can be plotted against $[\text{H}^+]$, for, if
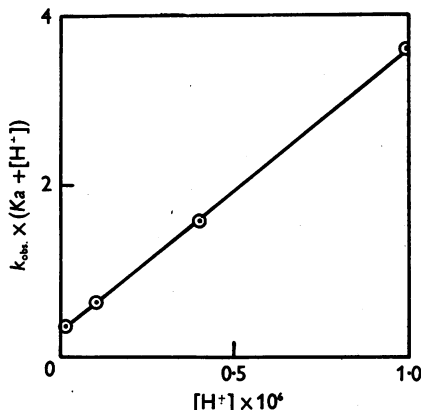

Fig. 3. Plot of $k_{obs.}$ $([\text{H}^+] + K_a)$ against hydrogen-ion concentration for the data at 24·6° in Fig. 2.

Eqn. iv holds, the graph should be linear. Fig. 3, which is the plot for the data at 24·6°, shows that this is so. Similar linear plots were obtained for the data at 11·7, 18·2 and 30·4°. This linearity supports the mechanism given by reactions 1 and 2 and the further analysis rests on this basis.

The slope of the line in Fig. 3 gives the value of $k_{HCN} = 3·26 \pm 0·02 \text{M}^{-1} \text{min.}^{-1}$ and the intercept could be used to obtain a value of the product $k_{CN^-} \cdot K_a$. However, a better value for this quantity with more accurately determined limits can be got by an alternative plot of the data. The function $k_{obs.}([\text{H}^+] + K_a)$ $[\text{H}^+]$ plotted against the reciprocal of $[\text{H}^+]$ is also linear and the slope in this case is given by $k_{CN^-} \cdot K_a$. Using a value of K_a calculated as described above, k_{CN^-} is found to be $914 \pm 24 \text{M}^{-1}$ sec.⁻¹ at 24·6°. The formation of the complex from the free cyanide ion thus proceeds about 280 times faster than the formation from the undissociated HCN molecule at this temperature. The calculated values of K_a and the values for k_{HCN} and k_{CN^-} at the four temperatures used are listed in Table 3.

Fig. 4 shows the appropriate plot to determine the activation energy E for the two reactions. The vertical lines within the circles in this diagram represent the spread of the experimental values.

Table 4 records values of these activation energies together with A, the temperature-independent factor, and ΔH^* and ΔS^*, the heat and entropy of activation for the two reactions. The value of A is

Table 3. *Velocity constants for ferricytochrome c-cyanide complex formation and ionization constants for HCN*

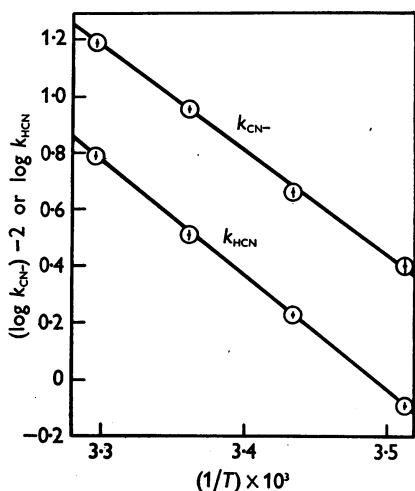| Temp. (°)	$K_a \times 10^{10}$	k_{HCN} (M^{-1} min.$^{-1}$)	k_{CN^-} (M^{-1} min.$^{-1}$)
11·7	1·64	0·825±0·015	253±12
18·2	2·51	1·72 ±0·03	465±15
24·6	3·57	3·26 ±0·02	914±24
30·4	5·21	6·28 ±0·04	1560±30

Fig. 4. Plots to determine the activation energies for the formation of the ferricytochrome c-cyanide complex from the free cyanide ion (upper line) and the undissociated hydrocyanic acid molecule (lower line).

obtained from the equation $k = Ae^{-E/RT}$; the heat of activation is given by $\Delta H^* = E - RT$ and the entropy of activation is calculated from the data at 24·6° using the equation

$$k = 1\cdot43 \times 10^{13} \exp\left(\frac{\Delta S^*}{R}\right) \exp\left(\frac{\Delta H^*}{RT}\right). \quad \text{(v)}$$

Examination of Fig. 2 now shows that the above mechanism for the formation of the complex does not hold over the entire pH range examined. If it did so, in alkaline solution when all the cyanide is present as CN$^-$, $k_{obs.}$ should tend asymptotically to the value of k_{CN^-}. It is clear that another ionization is operative, responsible for a diminution in the velocity of function of the complex. The ionization of HCN has already been taken into account, so this additional ionization must be attributed to the ferricytochrome c. The position of the maximum in Fig. 2 indicates roughly that the pK for this ionization lies between 8·5 and 9·2, having a bigger value the lower the temperature.

The simplest mechanism based on an ionization of this kind is to assume that the acid form of ferricytochrome c reacts with HCN and CN$^-$, giving the complex, and that the alkaline form does not react
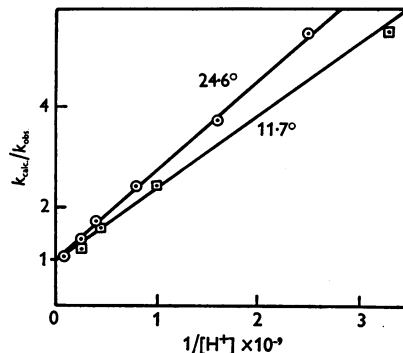

Fig. 5. Plot of the calculated velocity constant for the formation of the complex divided by the observed value as a function of $1/[\text{H}^+]$ at 11·7 and 24·6° based on the data in Fig. 2.

at all. If K_F is the ionization constant of ferricytochrome c, then the fraction of the acid form at any hydrogen-ion concentration will be given by $\frac{[\text{H}^+]}{K_F + [\text{H}^+]}$, and it follows that the relation between $k_{obs.}$, the observed velocity constant for the formation of the complex, and $k_{calc.}$, the value this velocity constant would have if the ionization did not occur, will be given by

$$k_{obs.} = k_{calc.} \frac{[\text{H}^+]}{K_F + [\text{H}^+]}. \quad \text{(vi)}$$

According to this equation, $k_{calc.}/k_{obs.}$ plotted against the reciprocal of $[\text{H}^+]$ should be linear. Fig. 5 shows the data for 24·6° and 11·7° plotted in this way. Quite good straight lines are obtained which thus support this simple ionization mechanism and it may be concluded that the assumption that

Table 4. *Kinetic data for ferricytochrome c-cyanide complex formation*

(For definition of symbols, see Eqn. v.)

Reacting species	k at 24·6° (M^{-1} sec.$^{-1}$)	E (kg.cal.)	A (M^{-1} sec.$^{-1}$)	ΔH^* (kg.cal.)	ΔS^* (e.u.)
HCN	0·0543	18·4±0·4	$2\cdot2 \times 10^{12}$	17·8±0·4	−5·9±1·4
CN$^-$	15·2	17·0±0·5	$5\cdot4 \times 10^{13}$	16·4±0·5	0·5±1·7

Table 5. *Acidity constants, heats and entropies of ionization for certain groups in proteins as compared with those of an ionizing group present in ferricytochrome c*

Group	Reference	pK	Temp. (°)	ΔH (kg.cal.)	ΔS (e.u.)
Phenolic OH (tyrosine)	1	9·8–10·4	25	6·0	− 24·4 to − 27·0
Glyoxaline (histidine)	1	5·6–7·4 (8·5)	25	6·9 to 7·5	− 0·3 to − 10·3 (− 15·3)
Glyoxaline (haemin-linked)	2	9·5	25	6·2	− 22·7
Haemin-water (in metmyoglobin)	3	8·85	25	3·85 ± 0·4	− 27·0 ± 2
Group in ferricytochrome c	4	8·75	24·6	2·9 ± 0·7	− 30 ± 3

(1) Cohn & Edsall (1943). The limit 8·5 in the pK for histidine-glyoxaline is taken from Theorell & Åkeson (1941 b). The ΔH and ΔS values are for both amino-acids and peptides and in general are experimental values at various ionic strengths.

(2) Russell & Pauling (1939). The ΔH value is that used by Wyman (1948).

(3) George & Hanania (unpublished results). The figures refer to an ionic strength similar to that used in the present investigation. The true thermodynamic values at 25° are nearer pK = 8·99, $\Delta H = 5·9 ± 0·7$ kg.cal. and $\Delta S = − 21 ± 2$ e.u.

(4) Present study.

Table 6. *Derived kinetic data for the dissociation of the ferricytochrome c-cyanide complex*

Dissociation into free cyt. +	k, at 24·6° (sec.$^{-1}$)	E (kg.cal.)	A	ΔH^* (kg.cal.)	ΔS^* (e.u.)
HCN	$1·13 \times 10^2$ M^{-1}	9·2 ± 1·0	$7·1 \times 10^8$	8·6 ± 1·0	− 21·6 ± 3·0
CN$^-$	$1·25 \times 10^{-5}$	15·9 ± 1·0	$6·25 \times 10^6$	15·3 ± 1·0	− 30·8 ± 3·0

the reaction of the alkaline form of ferricytochrome c giving the complex can be neglected was justified.

From Eqn. vi it can be seen that the slopes of these two lines give the values of K_F at the two temperatures. The values are $1·43 ± 0·07 \times 10^{-9}$ and $1·78 ± 0·02 \times 10^{-9}$ at 11·7 and 24·6°, respectively, corresponding to pK values of $8·84 ± 0·03$ and $8·75 ± 0·01$. Table 5 gives the heat and entropy of ionization calculated from these data, together with comparable data for the ionization of groups known to occur in proteins, and for the water molecule bound to the haemin group in metmyoglobin (see Eqn. 3).

$$Fe^+(H_2O) \rightleftharpoons FeOH + H^+. \qquad (3)$$
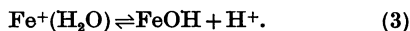
Using the values of k_{HCN} and k_{CN^-} obtained above, the equilibrium data recorded in Tables 1 and 2 can be analysed in the following way. The overall equilibrium constant K_{KCN} will be given by the equation

$$K_{KCN} = \frac{\dfrac{k_{HCN} \cdot [H^+]}{K_a + [H^+]} + \dfrac{k_{CN^-} \cdot K_a}{K_a + [H^+]}}{k_{diss.}}, \qquad (vii)$$

where $k_{diss.}$ is the velocity constant for the dissociation of the complex. The three constants in the numerator are known, and its numerical value has been calculated for each pH value in Tables 1 and 2. This divided by the respective value of K_{KCN} gives the corresponding value of $k_{diss.}$. In Fig. 6, $k_{diss.}$ obtained in this way is plotted against [H$^+$]. The straight lines obtained show that $k_{diss.}$ is compounded of a hydrogen ion-dependent dissociation and a normal dissociation. Inspection of reactions (1) and (2) shows that this was to be expected, for the corresponding back reactions would have these

characteristics. If k'_{HCN} and k'_{CN^-} represent the velocity constants for these back reactions, k'_{HCN} being the velocity constant at unit hydrogen-ion concentration, then

$$k_{diss.} = k'_{CN^-} + k'_{HCN} [H^+]. \qquad (viii)$$

The intercepts and slopes in Fig. 6 can thus be identified with k'_{CN^-} and k'_{HCN}. These constants have the values $2·7 \times 10^{-4}$ min.$^{-1}$ and $7·5 \times 10^{-4}$ min.$^{-1}$ at
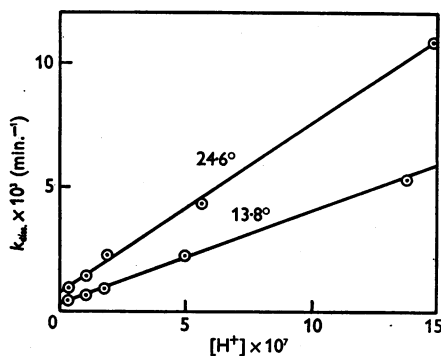

Fig. 6. Plot of the calculated dissociation velocity constant as a function of [H$^+$] at 13·8 and 24·6° based on kinetic data, including that in Fig. 2, and the equilibrium data recorded in Tables 1 and 2.

13·8 and 24·6°, and $3·78 \times 10^3$ M^{-1} min.$^{-1}$ and $6·8 \times 10^3$ M^{-1} min.$^{-1}$ at the same two temperatures, respectively. Table 6 lists the energies of activation, temperature-independent factors, heats and entropies of activation, calculated from these values in the same way as that used for the formation velocity constant data in Table 4. The individual equilibrium constants for reactions (1) and (2)

can now be calculated, for $K_1 = k_{HCN}/k'_{HCN}$ and $K_2 = k_{CN^-}/k'_{CN^-}$. In addition the change in heat content, ΔH, and the entropy change associated with the two reactions, may be obtained from the difference between the heats and entropies of activation of the forward and back reactions respectively. These data are listed in Table 7.

Table 7. *Derived equilibrium constants and overall heat and entropy changes for the formation of the ferricytochrome c-cyanide complex at 24·6°*

Reacting species	Equilibrium constants	ΔH (kg.cal.)	ΔS (e.u.)
HCN	$4\cdot8 \times 10^{-4}$	$9\cdot2 \pm 1\cdot4$	$+15\cdot7 \pm 4\cdot4$
CN$^-$	$1\cdot22 \times 10^6$ M^{-1}	$1\cdot1 \pm 1\cdot5$	$+31\cdot3 \pm 4\cdot7$

The complicated nature of the overall reaction revealed by this analysis shows that it would not have been possible to obtain reliable quantitative data from measurements of the equilibrium constant alone over a range of pH. Combining Eqn. vii with Eqn. viii the full expression for the overall equilibrium constant is

$$K_{KCN} = \frac{\dfrac{k_{HCN}\cdot[H^+]}{K_a + [H^+]} + \dfrac{k_{CN^-}\cdot K_a}{K_a + [H^+]}}{k'_{HCN}\cdot[H^+] + k'_{CN^-}}.$$

In acid solution this expression reduces to k_{HCN}/k'_{HCN} and so a value of K_1 could be obtained, but in solutions sufficiently alkaline to assume that K_1 is negligible, the ionization of the ferricytochrome c itself intervenes. At intermediate pH values a knowledge of individual velocity constants is essential for the analysis.

DISCUSSION

Horecker & Kornberg (1946) determined the velocity constants and the equilibrium constants over a pH range of 7·4–8. They found that these constants, when calculated on the basis of cyanide ion, remained constant within this pH range and they concluded, therefore, that ferricytochrome c reacted exclusively with cyanide ion. Results obtained in the present study have shown that ferricytochrome c reacts with both hydrocyanic acid and cyanide ion, the velocity constant for the latter reaction being about 280 times as large as that for the former reaction. The reaction with hydrocyanic acid is significant only at those pH values where cyanide is present predominantly as undissociated hydrocyanic acid.

Horecker & Kornberg's values for the velocity constant, the equilibrium constant and the activation energy for the cyanide ion reaction do not agree with ours. In part this is due to not taking into account the reaction with undissociated hydrocyanic acid and in part to using a different value for the

ionization constant of hydrocyanic acid. They do not state explicitly what value they have used, but in the caption to one of their figures they gave the free cyanide ion concentration at pH 7·4 in $4\cdot5 \times 10^{-3}$ M-potassium cyanide as $7\cdot9 \times 10^{-5}$ M-cyanide ion at 24°. This corresponds to a value for the ionization constant of hydrocyanic acid of $7\cdot0 \times 10^{-10}$ M in accord with the data of Madsen (1901). Using this and their value of 550 M^{-1} min.$^{-1}$ for k_{CN^-}, it can be shown that their experimental velocity constant would be about 9·5 M^{-1} min.$^{-1}$, whereas our data lead to a value of 11·4 M^{-1} min.$^{-1}$. There is thus far less numerical discrepancy between the experimental data than would appear from the determined constants, e.g. $k_{CN^-} = 914$ and 550 M^{-1} min.$^{-1}$ in our analysis and theirs, respectively. Their value for the activation energy, however, is widely different from ours, i.e. 26·1 kg.cal. as compared with our value of 17 kg.cal. We do not understand why this is so, except that it would arise if the fraction of free cyanide ion in potassium cyanide solution had been taken as independent of temperature. The difference between the activation energies corresponds closely to the heat of ionization of hydrocyanic acid of 9·8 kg.cal. (Madsen, 1901) or 10·8 kg.cal. (Harman & Worley, 1924) required on this supposition. The discrepancy between the values for the equilibrium constant, K_2, can also be accounted for in a similar way.

There is a very simple relationship which should hold between the two equilibrium constants for the formation of a complex through an ion and the corresponding undissociated acid. In the case of ferricytochrome c the cyanide complex is formed by reactions (1) and (2) (see p. 443). From the expressions for the two equilibrium constants, K_1 and K_2, it follows that $K_1/K_2 = K_a$, where K_a is the ionization constant for hydrocyanic acid. In Table 8 the

Table 8. *Relation between the equilibrium constants of the cyanide and hydrocyanic acid reactions with ferricytochrome c and the ionization constant of hydrocyanic acid*

Temp. (°)	13·8	24·6
Equilibrium constant of the HCN reaction, $K_1 \times 10^4$	2·72	4·8
Equilibrium constant of the CN$^-$ reaction, $K_2 \times 10^{-6}$	1·12	1·22
$K_1/K_2 \times 10^{10}$	2·43	3·93
Ionization constant of HCN $\times 10^{10}$	1·90	3·57

values of K_1/K_2 from the data obtained at 13·8 and 24·6° are compared with K_a. The agreement is very satisfactory in view of the lengthy derivation of the two equilibrium constants. The differences between the overall heats and entropies of the formation of the complex which are listed in Table 7 are also a consequence of this relationship between the two equilibrium constants. The differences should

correspond to the heat and entropy of ionization of hydrocyanic acid, i.e. $\Delta H = 10 \cdot 8$ kg.cal. and $\Delta S = + 6 \cdot 4$ e.u. (entropy units), which can be seen to be the case within the rather wide limits of the experimental uncertainty associated with these derived quantities.

The peculiar features of the formation of this cyanide complex can best be shown by the data for its formation from the cyanide ion by reaction (2). It is formed in a slightly endothermic reaction, $\Delta H = 1 \cdot 1 \pm 1 \cdot 5$ kg.cal. (Table 7), yet the activation energies for the forward and back reactions are quite large, $17 \cdot 0 \pm 0 \cdot 5$ and $15 \cdot 9 \pm 1 \cdot 0$ kg.cal. respectively (Tables 4 and 6). An activation energy of this magnitude is not unreasonable for the back reaction which involves the breaking of a Fe-CN bond, but the high activation energy for the forward reaction suggests that a strong bond has to be broken to allow the cyanide complex to form.

The overall entropy of formation of the complex in reaction (2) has a very large positive value, $\Delta S = 31 \cdot 3 \pm 4 \cdot 7$ e.u. (Table 7). Now in this reaction the cyanide ion is bound, and some part of its total entropy of $+25$ e.u. will be lost (Latimer, 1938). This might easily be about 10 e.u. and so, when the cyanide complex forms, an entropy change of about $+40$ e.u. must arise from changes in structure in the ferricytochrome c itself. This is very suggestive that the haematin is bound on both sides of the iron atom, for the high activation energy referred to above would be required to free one of the iron valencies, and the large positive entropy change would result from the more random arrangement of that part of the protein molecule previously bound to the iron atom. Such a model for cytochrome c has been discussed by Theorell (1941).

The dissociation of the complex is necessarily attended by a large negative entropy of activation, $-30 \cdot 8 \pm 3 \cdot 0$ e.u. (Table 6). This contributes to a great extent to the low value for the dissociation velocity constant, which has a low temperature-independent factor of $6 \cdot 25 \times 10^6$, compared with those for normal reactions of about 10^{13}. In fact the existence of the complex can be regarded as a consequence of this low dissociation velocity constant. The formation velocity constant, apart from the high activation energy, is normal in that the temperature-independent factor is about 10^{13} (Table 4). This reaction may be contrasted with other haemoprotein reactions such as that between haemoglobin and oxygen, when the existence of oxyhaemoglobin is not a consequence of a slow dissociation velocity constant but of an extremely large formation velocity constant. It is an attractive hypothesis to suppose that the orientation and binding of the haem or haematin groups is responsible for these differences. Hanania, George & Irvine (unpublished results) have recently observed that the formation of the metmyoglobin-cyanide complex is comparatively slow. The observed bimolecular constant at pH 7·0 and 18° is about $80 \, \text{M}^{-1} \, \text{sec.}^{-1}$ compared with the value $0 \cdot 037 \, \text{M}^{-1} \, \text{sec.}^{-1}$ for ferricytochrome c under the same conditions. This suggests that simple structural considerations alone cannot account for differences in reactivity.

Kinetic studies in alkaline solutions indicate that the reaction between ferricytochrome c and cyanide is affected by an ionizing group in the vicinity of the prosthetic group of the former. The dissociated form of ferricytochrome c reacts with cyanide, if at all, much more slowly than does undissociated ferricytochrome c with cyanide ion.

This ionizing group in ferricytochrome c which affects the formation velocity of the cyanide complex is of particular interest in connexion with the binding of the haemin-iron atom to the protein. No ionizing group with precisely the same pK value has been revealed in other investigations. By oxidation-reduction potential studies, Rodkey & Ball (1950) discovered an ionizing group with a pK value of 7·8 and by spectrophotometric titration, Theorell & Åkeson (1941b) showed that an ionizing group, with a pK of 9·35, is responsible for the change in absorption spectrum of ferricytochrome c from types III to IV. The pK value obtained in the present study is sufficiently different from that of Rodkey & Ball to exclude identification of the two groups concerned. On the other hand, it seems to be possible that the ionizing group responsible for the change in absorption spectrum obtained by Theorell & Åkeson might be the same group described in the present work. The discrepancy in pK values could be attributed to the differences in ionic strength of the ferricytochrome c solutions used, and it is not inconceivable that cytochrome c preparations with an iron content of 0·34 % used in the present work might behave slightly differently from the preparation of iron content 0·43 % employed by Theorell & Åkeson.
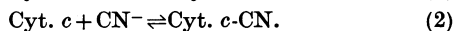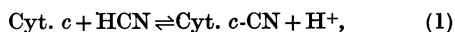
By titration studies, Theorell & Åkeson (1941c) reached the conclusion that only one of the three histidine molecules present in cytochrome c was titrated within its normal pH range and two haemin-linked acid groups were titrated between pH 9 and 10. It was argued therefore that these two haemin-linked groups were both histidine glyoxaline groups. This theory has also received the support of Paul, who succeeded in splitting off the cytochrome c prosthetic group from the protein (Paul, 1950), and in a brief communication (Paul, 1949) also reported that between pH 4 and 6 two more acid groups were titrated in the protein residue than in the intact cytochrome molecule. However, no details of the latter findings have yet been made available.

Unfortunately, the heat of ionization of Theorell & Åkeson's haemin-linked acid groups was not

reported. The heat and entropy of ionization of the group described in the present work seem to be rather different from what would be expected from a histidine glyoxaline residue, although it is not known whether haemin-linked glyoxaline groups might have slightly different values. The pK value of 9·5 reported by Russell & Pauling (1939) for glyoxaline in the methaemoglobin-glyoxaline complex is an indication that this may be so, but again, the values of ΔH and ΔS are not available, and so a full comparison is not yet possible. It is interesting to point out, however, that the heat of ionization for the group in cytochrome c is particularly low, i.e. $2·9 \pm 0·7$ kg.cal., whereas weak acids with a pK in this range usually have heats of the order of 10–12 kg.cal. (Cohn & Edsall, 1943).

SUMMARY

1. A kinetic investigation of the formation of the ferricytochrome c-cyanide complex over the pH range 6·0–9·0 suggests that both un-ionized hydrocyanic acid and the free cyanide ion react:

$$\text{Cyt. } c + \text{HCN} \rightleftharpoons \text{Cyt. } c\text{-CN} + \text{H}^+, \quad (1)$$
$$\text{Cyt. } c + \text{CN}^- \rightleftharpoons \text{Cyt. } c\text{-CN}. \quad (2)$$

Analysing the data according to this mechanism, the bimolecular constants k_{HCN} and $k_{\text{CN}-}$ were found to be $5·43 \times 10^{-2}$ and $15·2\text{M}^{-1}$ sec.$^{-1}$ respectively, at 24·6°. From experimental data at various temperatures, the activation energies were determined as $18·4 \pm 0·4$ and $17·0 \pm 0·5$ kg.cal. respectively.

2. Using these velocity constants and equilibrium data obtained over the pH range 5·8–7·5 the dissociation velocity constants were calculated and found to be of the form

$$k_{\text{diss.}} = k'_{\text{CN}-} + k'_{\text{HCN}} [\text{H}^+],$$

where $k'_{\text{CN}-}$ and k'_{HCN} can be identified as the reverse of reactions (2) and (1) respectively. At 24·6°, $k'_{\text{CN}-}$ and k'_{HCN} are $1·25 \times 10^{-5}$ sec.$^{-1}$ and $1·13 \times 10^2 \text{M}^{-1}$ sec.$^{-1}$ respectively and the activation energies $15·9 \pm 1·0$ and $9·2 \pm 1·0$ kg.cal.

3. Combining these kinetic data gives the values for the equilibrium constants for reactions (1) and (2), $K_1 = 4·8 \times 10^{-4}$ and $K_2 = 1·22 \times 10^6 \text{M}^{-1}$. The corresponding changes in heat content and entropy are $9·2 \pm 1·4$ kg.cal. and $+15·7 \pm 4·4$ e.u.; and $1·1 \pm 1·5$ kg.cal. and $+31·3 \pm 4·7$ e.u. respectively. For such reactions the relationship $K_1/K_2 = K_a$ should hold, where K_a is the dissociation constant of hydrocyanic acid: this was found to be so within the experimental error.

4. Kinetic experiments in alkaline solutions, pH 9–10, showed that the velocity constant for the formation of the complex decreases, which may be attributed to an ionization on the ferricytochrome c molecule such that the formation of the complex occurs predominantly by reaction of the acid form.

5. The pK for this ionization was calculated as 8·75 at 24·6° and the heat and entropy of ionization were found to be $2·9 \pm 0·7$ kg.cal. and -30 ± 3 e.u. respectively.

6. The quantitative data given above support a model for ferricytochrome c in which the haematin iron atom is firmly bound on both sides to the protein.

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