Table 4. *Phosphatase activity of small intestine of rats on different diets*

(Ten rats on each diet. Results (mean ± S.E.) expressed as 'units', i.e. mg. P liberated in 1 hr. at pH 9·2 and 37°.)

Tissue		Diet: Basal	Fat-free	High fat	High protein
Whole intestine	Total units	(1) 330±46·5	(2) 391±41·7	(3) 489±39·3	(4) 538±42·6
	Units/g.	(5) 52±7·1	(6) 58±4·8	(7) 77±6·3	(8) 78±6·4
Proximal portion	Total units	(9) 271±40·7	(10) 328±31·3	(11) 375±33·0	(12) 448±37·2
	Units/g.	(13) 77±10·6	(14) 87±6·0	(15) 113±8·3	(16) 119±10·9
Distal portion	Total units	(17) 58±7·8	(18) 62±13·3	(19) 114±10·8	(20) 90±16·1
	Units/g.	(21) 20·3±3·4	(22) 20·3±3·9	(23) 34·5±3·5	(24) 29·0±3·7

Significant differences ($P < 0.05$) exist between the following groups: (1) and (3), (1) and (4), (2) and (4), (5) and (7), (5) and (8), (6) and (7), (6) and (8), (9) and (11), (9) and (12), (10) and (12), (13) and (15), (13) and (16), (14) and (15), (14) and (16), (17) and (19), (17) and (20), (18) and (19), (21) and (23), (22) and (23).

DISCUSSION

The chief difference between the basal and fat-free diets on the one hand and the high fat and the high protein diets on the other is the presence of much carbohydrate (55 or 70% sucrose) in the former and its absence in the latter. It would seem therefore that the presence of sucrose leads to a reduction in the amount of phosphatase in the intestinal tissue. References to similar work are very meagre. Bellini & Cera (1940) found an increase in the intestinal phosphatase of the rat during fat absorption. Weil & Russell (1940) found a decrease in plasma phosphatase in the rat during fasting, and an increase during the feeding of lard and of cephalin but not of lecithin. Lundbaek & Goranson (1948) showed that the phosphorylase of rat muscle is increased during starvation.

We can at present suggest no reason why the presence of sucrose in the diet should lead to a decrease in intestinal phosphatase, and until more data are available it is profitless to speculate.

SUMMARY

The effect was studied of varying the proportions of dietary protein, fat and carbohydrate on the alkaline phosphatase of the small intestine of the rat. Diets containing 55 or 70% of sucrose produced a significantly lower amount of the enzyme than diets in which the sucrose was replaced by fat or protein.

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The Reactions of Haems with Cyanides and *iso*Cyanides

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By determining the minimum amount of pyridine required to give a haemochromogen with a very strong solution of haem (ferrous protoporphyrin), Hill (1926) demonstrated conclusively that pyridine haemochromogen contains 2 molecules of organic base per molecule of haem or per atom of iron. This result was in agreement with that of von Zeynek (1910) who found 2·2 mol. of pyridine per mol. of haem in a solid pyridine haemochromogen.

Potassium cyanide, however, unlike other nitrogenous substances, was found by Anson & Mirsky (1928) to give two distinct compounds with haem. The first, containing 1 mol. of cyanide per molecule of haem, was named by them 'cyan-haemochromogen'

on account of the resemblance of its absorption spectrum to that of pyridine haemochromogen. The second compound, containing more cyanide per molecule of haem, was merely named the 'second cyanide compound'. The latter, which can only be obtained in presence of an excess of cyanide, differs from the first in the general pattern of its absorption spectrum. Unlike a typical haemochromogen its β-band is much stronger than its α-band. (Following general usage the letters α, β and γ denote the individual bands of the absorption spectrum of a compound, proceeding in that order from the red towards the ultraviolet regions of the spectrum. In haematin compounds the α- and β-bands are in the visible region; while the γ-band, also known as the Soret band, lies in the violet part of the spectrum.) These facts led Anson & Mirsky (1928) to consider all typical haemochromogens as compounds of haem with 1 mol. of a nitrogenous base, a conclusion which was not corroborated by later work. The nature of the compounds of cyanide with haem was reinvestigated by Hill (1929), who confirmed Anson & Mirsky's finding that haem and cyanide may combine in equimolecular proportions to give a compound with a haemochromogen-like spectrum, especially if the solution has a relatively low concentration of salts which tend to precipitate the haem out of solution. He also showed that the second compound, which had previously been obtained in the presence of excess cyanide, contains only 2 mol. of cyanide per molecule of haem, and he was able to determine the dissociation constants of both compounds.

As it is the 'second cyanide compound' of Anson & Mirsky (containing 2 molecules of cyanide per molecule of haem) that is structurally analogous to pyridine haemochromogen, Hill described it under the name 'cyan-haemochromogen' whereas for the monocyanide compound he proposed the name 'cyan-reduced-haematin'. Since, as will be shown later, both these cyanide-haem compounds differ in several respects from a typical haemochromogen, the protohaem compounds containing 1 and 2 mol. of cyanide will be described as monocyan-haem [(CN^-)-haem] and dicyan-haem [$(CN^-)_2$-haem] respectively. Suggested structures for these two compounds are

$$K^+ [\text{Porphyrin } Fe^{II} (CN)]^-$$

and $$K_2^{++} [\text{Porphyrin } Fe^{II} (CN)_2]^{--}.$$

In this paper it is proposed (1) to give the absorption spectra in the visible and violet regions of monocyan- and dicyan-haem; (2) to answer the question raised by Hill (1926) as to the existence of a compound in which the haem iron is linked simultaneously to the cyanide radical and carbon monoxide; (3) to elucidate the mode of attachment of the cyanide ion to the haem iron, since it is theoretically possible for the haem iron to combine with either the nitrogen or the carbon atom of the cyanide radical. For this the reactions of non-dissociable cyanides and *iso*cyanides with haems, haematins and methaemoglobin were examined.

EXPERIMENTAL

Protohaemin. This was prepared by the method of Schalfejeff (1885) from ox or horse blood.

Urohaemin. Uroporphyrin I was isolated from the urine of a case of congenital porphyria. The porphyrin was esterified and the urohaemin prepared from the octamethyl ester according to Fischer & Orth (1934).

Other reagents. KCN solutions in distilled water were standardized either by the method of Robbie & Leinfelder (1945) or with standard $AgNO_3$ in the usual manner. Methyl cyanide was purified by adapting the method of Toda (1926) for the purification of butyl cyanide. Methyl *iso*cyanide was prepared according to the method of Gautier as modified by Hartley (1928).

Spectroscopic observations. For all qualitative spectroscopic examinations and for preliminary quantitative experiments a microspectroscope was used as previously described (Keilin, 1943). The detailed study of absorption spectra was carried out with a Beckman photoelectric spectrophotometer using 0·5 and 0·25 cm. cells. The absorption coefficient is defined as $\epsilon = d/cl$, where c = molarity of the haematin solution, l = optical depth and d (density) = $\log I_0/I$, where I_0 and I are the intensities of the incident and transmitted light, respectively.

RESULTS

Monocyan-haem

A stock solution of protohaematin ($1{\cdot}8 \times 10^{-4}$ M) was prepared by dissolving 11·7 mg. crystalline protohaemin in 100 ml. 1% (w/v) (anhydrous) Na_2CO_3. Monocyan-haem [(CN^-)-haem] was obtained by mixing 1 ml. of the stock haematin solution with 0·5 ml. of $3{\cdot}6 \times 10^{-4}$ M aqueous solution of potassium cyanide, the mixture being made up to 6 ml. with water and reduced with a few mg. of solid sodium dithionite ($Na_2S_2O_4$). In this solution the haem and cyanide were present in a 1:1 molecular ratio, the concentration of each being $0{\cdot}3 \times 10^{-4}$ M. The absorption spectrum of the scarlet solution of this compound shows two distinct bands in the visible region: α, 552 and β, 522 mμ., the α-band being much stronger than the β-band. The γ-band is at 414 mμ. The degree of dissociation which occurred under the conditions of this experiment was calculated from the value of the dissociation constant $K = 1{\cdot}3 \times 10^{-5}$ at 16°, given by Hill (1929). It was found that only 52·4% of the total haem was present as (CN^-)-haem, 47·6% remaining as free haem. These figures are in agreement with Hill's dissociation curve of (CN^-)-haem where cyanide and haem are present in a 1:1 molecular ratio. The absorption curve of (CN^-)-haem, as measured under the above experimental conditions, and the theoretical

curve of the absorption spectrum for 100 % (CN⁻)-haem, which was calculated from the observed values, are shown in Figs. 1 and 2.

The extinction coefficients of the α-, β- and γ-bands, measured and theoretical, are summarized in Tables 1 and 2.

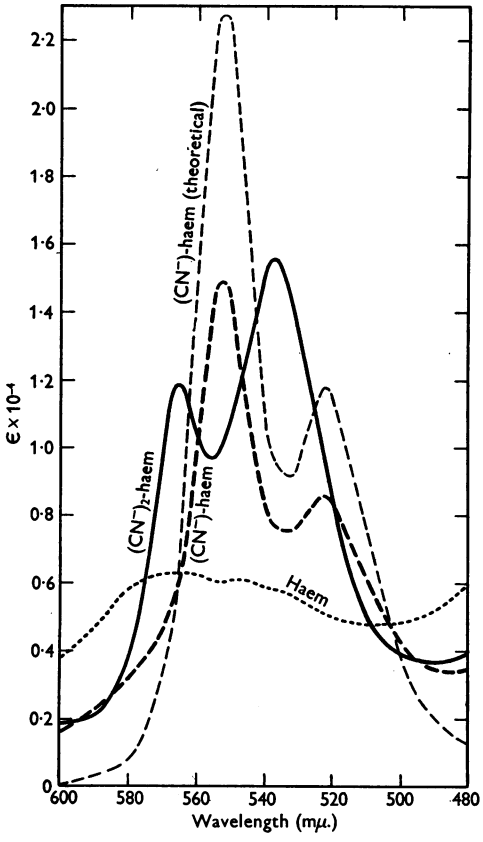


Fig. 1. Absorption bands in the visible region of protohaem, (CN⁻)-protohaem and $(CN^-)_2$-protohaem. Haem = 0·3 × 10^{-4} M, l = 0·5 cm., ϵ as defined in text. The theoretical absorption curve of (CN⁻)-haem calculated for the undissociated compound is also given.

The absorption spectrum of this compound is of special interest in that although it is of a haemochromogen pattern, its α- and β-bands are considerably lower than those of typical haemochromogens obtained with denatured serum proteins, denatured globin, glycine or pyridine (Table 1). However, in the case of (CN⁻)-urohaem (Fig. 5) it was found that the α-band was very nearly as high ($\epsilon \times 10^{-4} = 1{\cdot}99$) as that of a typical urohaemochromogen ($\epsilon \times 10^{-4} = 2{\cdot}23$ for glycine urohaemochromogen).

So far, the magnetic susceptibility of (CN⁻)-haem has not been determined owing to technical difficulties. As has already been pointed out by Hill (1929), the strongest solution of (CN⁻)-haem that can be obtained is 2×10^{-4} M. Even though the haem and cyanide are present in 1:1 ratio, a more concentrated solution would contain free haem in equilibrium with dicyan-haem. For the accurate determination of the magnetic susceptibility of a haematin derivative by the method of Gouy (1889) using a magnetic field of about 10,000 gauss, the concentration of the haem should be at least 10^{-2} M.

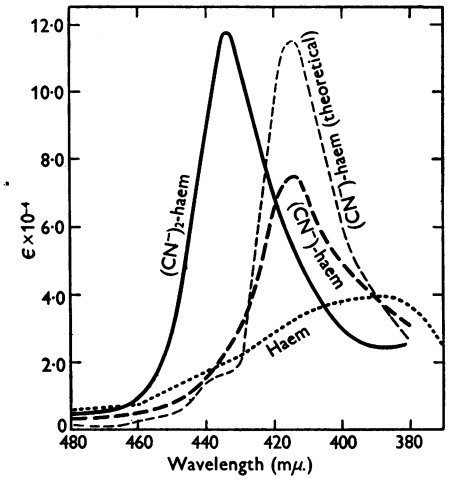


Fig. 2. Absorption spectra showing γ-bands of protohaem, (CN⁻)-protohaem and $(CN^-)_2$-protohaem, and the theoretical absorption curve of (CN⁻)-protohaem calculated for the undissociated compound. Haem = 0·3 × 10^{-4} M, l = 0·5 cm.

Dicyan-haem

Dicyan-haem, [$(CN^-)_2$-haem], was prepared by mixing 1 ml. of the stock haematin solution with 5 ml. of $3{\cdot}6 \times 10^{-3}$ M aqueous potassium cyanide solution reduced with the minimum amount of dry $Na_2S_2O_4$. The concentration of haem was $0{\cdot}3 \times 10^{-4}$ M and the haem:cyanide ratio was 1:100. The absorption spectrum of the solution of this compound, which is also scarlet, differs markedly from that of (CN⁻)-haem (Figs. 1 and 2). The positions of its two absorption bands in the visible region of the spectrum are: α, 565 and β, 536 mμ., and the α-band is much weaker than the β-band, the values $\epsilon \times 10^{-4}$ being 1·185 and 1·56, respectively. The γ-band lies at 434 mμ. The extinction coefficients of the absorption bands are summarized in Table 2.

Reactions with carbon monoxide

Reactions of haemochromogens with carbon monoxide. It is well known that haem and haemochromogens combine reversibly with CO and that the CO-haem and CO-haemochromogens thus formed each contain only 1 mol. of CO/mol. of the compound

Table 1. *Positions and heights of the α- and β-bands and the trough between them for monocyan-protohaem and typical haemochromogen compounds*

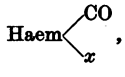
Compound	Band or trough (tr.)	Wavelength of maxima and minima of absorption (mμ.)	$\epsilon \times 10^{-4}$	$\frac{\epsilon_\alpha}{\epsilon_{tr.}}$	$\frac{\epsilon_\beta}{\epsilon_{tr.}}$
Monocyan-protohaem (theoretical value)	α	552	2·27		
	tr.	534	0·91	2·5	1·3
	β	522	1·18		
Denatured serum protein haemochromogen (Keilin, 1944)	α	557	2·96		
	tr.	540	0·79	3·76	1·64
	β	527	1·29		
Glycine haemochromogen (Keilin, unpublished)	α	556	3·02		
	tr.	539	0·83	3·65	1·71
	β	525	1·30		
Globin haemochromogen (Drabkin, 1942)	α	558	3·09		
	tr.	542	0·89	3·49	1·58
	β	528	1·40		
Pyridine haemochromogen (Drabkin, 1942)	α	558	3·09		
	tr.	540	0·91	3·41	1·79
	β	525	1·63		

Table 2. *Positions and extinction coefficients of the α-, β- and γ-bands of the compounds of protohaem and urohaem with* KCN, CH_3NC *and* CO

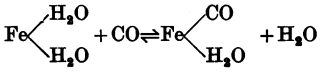
Compound	α-Band λ (mμ.)	α-Band $\epsilon \times 10^{-4}$	β-Band λ (mμ.)	β-Band $\epsilon \times 10^{-4}$	γ-Band λ (mμ.)	γ-Band $\epsilon \times 10^{-4}$	$\frac{\epsilon_\gamma}{\epsilon_\alpha}$
(CN^-)-protohaem (theoretical)	552	2·27	522	1·18	414	11·45	5
(CN^-)-protohaem (exp.)	552	1·49*	522	0·86*	414	7·5*	5
(CH_3NC)-protohaem	556	1·515*	528	1·13*	420	10·45*	7
$(CN^-)_2$-protohaem	565	1·185	536	1·56	434	11·75	10·0
$(CH_3NC)_2$-protohaem	562	1·245	532	1·415	432	14·48	11·6
CO-protohaem	562	1·46	530	1·195	406·5	14·7	9·6
(CN^-)-protohaem + CO	562	1·40	530	1·15	406·5	14·1	10·0
CO-cyan-protohaem	565	0·97	538	1·185	425	15·0	15·4
CO-protohaem (in ethanol)	562	1·185	533	0·92	410	15·6	13·2
CO-(CH_3NC)-protohaem	564	1·205	536	1·155	422·5	10·0	8·3
(CN^-)-urohaem	547	1·99*	518	1·17*	418	11·2*	5·6
(CH_3NC)-urohaem	547	1·655*	516	1·185*	410	17·5*	10·5
$(CN^-)_2$-urohaem	560	0·83	532	1·46	428	11·0	13·25
$(CH_3NC)_2$-urohaem	554	1·1	524	1·55	422	26·8	24·5

* These values apply to the compound under the experimental conditions described and are much lower than the theoretical value owing to dissociation.

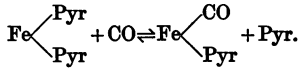
(Hill, 1926). According to Hill CO-haem can be represented as



'where x is either another molecule of the complex or a molecule of water'. (In the schematic representations of these compounds, the lines directed from the haem iron are not intended to indicate the nature of the valency bonds.) Using the formula for haem suggested by Davies (1940) the reaction may be expressed as follows (Fe = haem iron):



By passing CO through a solution of a haemochromogen, e.g. pyridine haemochromogen, the CO displaces 1 mol. of pyridine (Pyr) to form a CO-haemochromogen (Hill, 1926):



It is important to note that in this equilibrium reaction CO and pyridine compete for the haem iron.

Reaction of monocyan-haem with carbon monoxide. On passing CO through a solution of (CN^-)-haem, the latter acquires a characteristic bright cherry-red colour, and its absorption spectrum in both the visible and violet regions becomes almost

indistinguishable from that of ordinary CO-haem. To avoid loss of cyanide as CO is bubbled through the solution, the experiment was also carried out by adding an equivalent amount of cyanide to CO-haem previously prepared. However, the same result was obtained in both cases and the compound formed could not be distinguished from CO-haem. These results support the observation of Hill (1929) that when (CN⁻)-haem is treated with CO the latter replaces the single cyanide ion giving CO-haem and not CO-(CN⁻)-haem. The reaction may therefore be represented as follows:

$$\text{Fe}\begin{matrix}\text{CN}^- \\ \text{H}_2\text{O}\end{matrix} + \text{CO} \rightleftharpoons \text{Fe}\begin{matrix}\text{CO} \\ \text{H}_2\text{O}\end{matrix} + \text{CN}^-$$

Reaction of dicyan-haem with carbon monoxide. When CO was passed through a solution of $(CN^-)_2$-haem, the characteristic absorption bands of this compound in the visible region of the spectrum disappeared and were replaced by a diffuse and asymmetric band as seen in the microspectroscope. The spectrophotometric curve of this band (Fig. 3), however, showed it to consist of a two-banded spectrum with a very weak and narrow band at 565 mμ. and a strong band at 538 mμ. There was also a very marked change in the violet region of the spectrum; the γ-band of the compound became distinctly sharper and was shifted from 434 to 425 mμ., i.e. about 9 mμ. towards the short wave end of the spectrum (Fig. 4, Table 2). The absorption spectrum of this compound is thus very different from those of (CN⁻)-haem, $(CN^-)_2$-haem, CO-haem and CO-haemochromogen. These results clearly demonstrate the existence of CO-cyan-haem, which may be considered as analogous to CO-haemochromogens:

$$\text{Fe}\begin{matrix}\text{CN}^- \\ \text{CN}^-\end{matrix} + \text{CO} \rightleftharpoons \text{Fe}\begin{matrix}\text{CO} \\ \text{CN}^-\end{matrix} + \text{CN}^-.$$

Carbylamine haems

Reactions of methyl isocyanide with haems. Pure methyl *iso*cyanide (CH_3NC) was dissolved in distilled water to make a 0·15 M solution. A 10^{-3} M solution of protohaematin was prepared by dissolving 16·4 mg. of crystalline protohaemin in 25 ml. 0·1 N-NaOH. The *iso*cyanide solution (1 ml.) was added to 1 ml. haematin solution, and the mixture was diluted with methanol and distilled water so that the final methanol concentration was at least 50 %. When this solution, containing haem and *iso*cyanide in a 1:150 molecular ratio, was reduced with $Na_2S_2O_4$ the red solution showed a two-banded spectrum resembling that of $(CN^-)_2$-haem, the β-band being sharper than the α-band. The positions of the bands were: α, 562, β, 532 and γ, 432 mμ., the values of $\epsilon \times 10^{-4}$ being 1·25, 1·42 and 14·48, respectively (Table 2). A similar experiment using equimolecular (10^{-3} M) solutions of haematin and *iso*cyanide gave rise to a spectrum resembling that of (CN⁻)-haem in

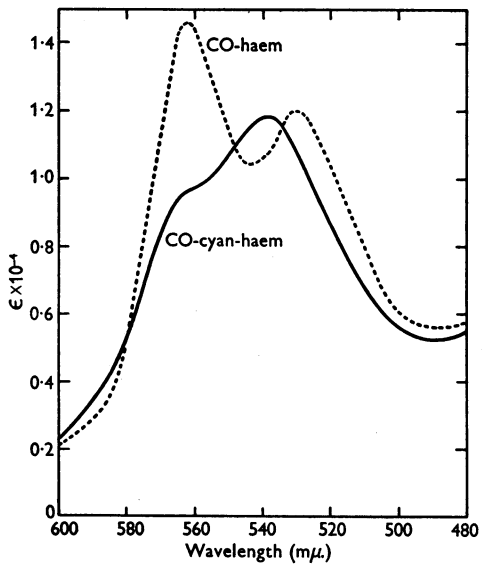


Fig. 3. Absorption bands in the visible region of the spectrum of CO-protohaem and CO-cyan-protohaem. Haem = $0{\cdot}3 \times 10^{-4}$ M, $l = 0{\cdot}5$ cm.

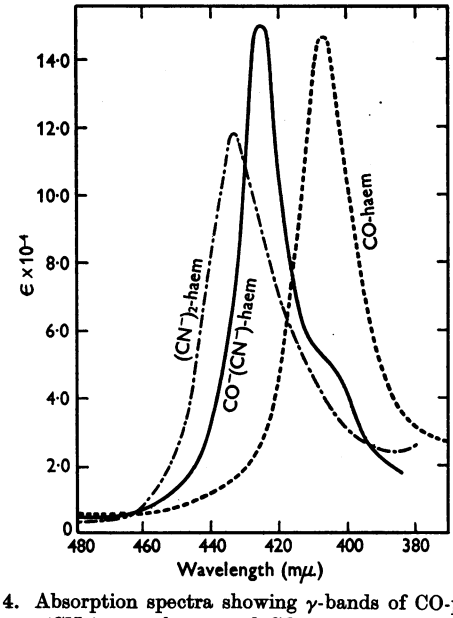


Fig. 4. Absorption spectra showing γ-bands of CO-protohaem, $(CN^-)_2$-protohaem and CO-cyan-protohaem. The shoulder at about 405 mμ. on the latter absorption band is probably due to a little CO-haem in the solution. Haem = $0{\cdot}3 \times 10^{-4}$ M, $l = 0{\cdot}5$ cm.

which the α-band was much sharper than the β-band. Owing to some dissociation under the conditions of the experiment the absorption spectrum is

probably that of a mixture of (CH_3NC)-haem with a little free haem and (CH_3NC)$_2$-haem, the latter giving rise to a low γ-band in addition to that of (CH_3NC)-haem. The positions and extinction coefficients of the absorption bands of this solution are summarized in Table 2.

With urohaem, CH_3NC reacts freely in slightly alkaline solution, presumably because urohaem is so much more soluble than protohaem. Two compounds are formed as with protohaem, and the spectra are

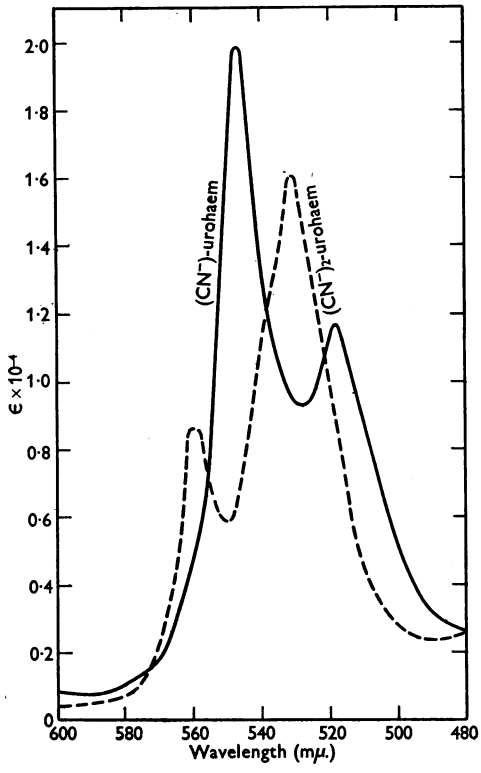


Fig. 5. Absorption bands in the visible region of the spectrum of (CN^-)-urohaem (Haem:KCN = 1:3·6) and (CN^-)$_2$-urohaem, containing excess KCN. Urohaem = 0·55 × 10^{-4} M for first compound and 0·661 × 10^{-4} M for second compound; l = 0·25 cm. The γ-bands of these compounds are shown in Fig. 7.

very similar to those of mono- and di-cyan-urohaem. (CH_3NC)-urohaem has its α-, β- and γ-absorption bands at 547, 516 and 410 mμ., the values of $\epsilon \times 10^{-4}$ being 1·66, 1·19 and 17·5, respectively (Figs. 6 and 7).

(CH_3NC)$_2$-Urohaem has α- and β-bands at 554 and 524 mμ., the values of $\epsilon \times 10^{-4}$ being 1·1 and 1·55, respectively. The γ-band of this compound, which lies at 422 mμ., is of interest in that the value of $\epsilon \times 10^{-4}$ is about 26·8 (certainly above 25·6) (Figs. 6 and 7). The only other metalloporphyrin known which possesses such a high γ-band is turacin, a naturally occurring copper-uroporphyrin. In turacin the γ-band is at 399 mμ. and $\epsilon \times 10^{-4}$ is 26.

The reaction between protohaem and 10^{-2}M-CH_3NC in alcoholic solution with cysteine buffer as reducing agent was described by Warburg, Negelein & Christian (1929) in the course of their investigations on the compound formed between methyl *iso*cyanide and haemoglobin and on the effects of methyl *iso*cyanide on the photochemical dissociation of CO-haemoglobin. These authors give the absolute absorption spectrum of methyl carbylamine-haem of which only the γ-band resembles that of (CH_3NC)$_2$-urohaem as shown in Fig. 7. The mono-carbylamine-haem compound was not described by these authors.

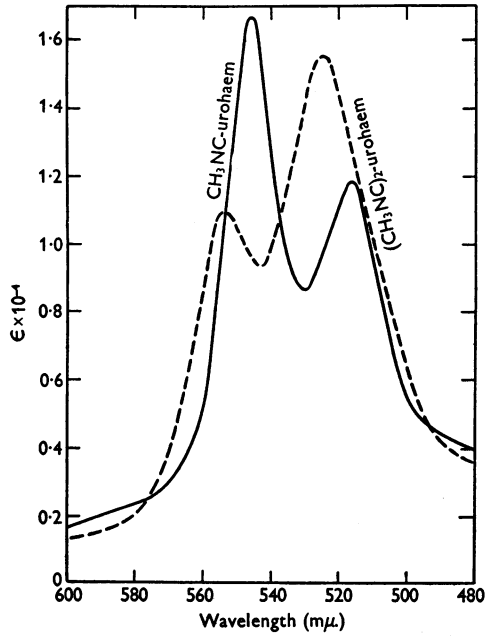


Fig. 6. Absorption bands in the visible region of the spectrum of CH_3NC-urohaem (urohaem:CH_3NC = 1:1) and (CH_3NC)$_2$-urohaem (urohaem:CH_3NC = 1:150). Urohaem = 0·834 × 10^{-4} M, l = 0·25 cm.

Reactions of carbon monoxide with mono- and di-carbylamine haems. With (CH_3NC)-protohaem, as in the case of (CN^-)-protohaem, CO displaces the single molecule of CH_3NC from the haem Fe, and ordinary CO-haem is obtained. When CH_3NC was added to an ethanolic solution of CO-protohaem so that the molecular ratio of CH_3NC to haem was 3:1, the α- and β-bands became more diffuse and a γ-band appeared at 422·5 mμ. in addition to that of CO-haem (410 mμ.). This new band, which lay in a position intermediate between those of CO-haem and (CH_3NC)$_2$-haem, was most probably due to a CO-carbylamine-haem compound, analogous to CO-(CN^-)-haem. The same result is obtained if CO is

passed through a solution of $(CH_3NC)_2$-haem containing CH_3NC and haem in the same 3:1 ratio. If more CH_3NC is present, e.g. 15 mol./mol. of haem, the CO compound does not appear to be formed.

Reactions of methyl cyanide with haem. Whereas a commercial sample of methyl cyanide (CH_3CN) reacted with urohaem giving a 4-banded spectrum corresponding to a mixture of the mono- and di-cyan-haem types of spectra, a pure specimen (b.p. 81°) which had been treated with N-H_2SO_4 to hydrolyse any *iso*cyanide present, followed by N-NaOH to remove any free HCN, failed to react with urohaem in any proportions in both aqueous and ethanolic solutions. The importance of this failure to react with haem will be discussed later.

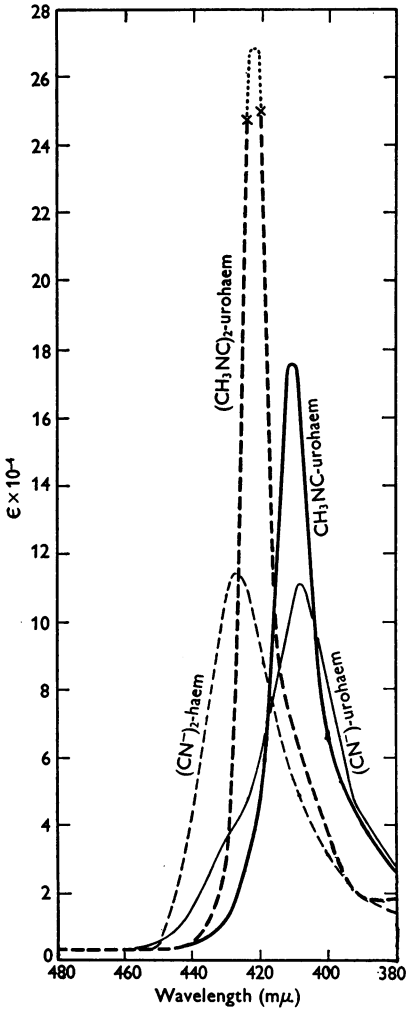


Fig. 7. Absorption spectra showing γ-bands of $(CN^-)_2$-urohaem, (CN^-)-urohaem (solutions as in Fig. 5), $(CH_3NC)_2$-urohaem and CH_3NC-urohaem (solutions as in Fig. 6). $l = 0{\cdot}25$ cm.

Reactions of methyl isocyanide and methyl cyanide with the trivalent iron compounds, haematin and methaemoglobin

Haematin. KCN reacts readily with haematins causing the colour to change from greenish brown to scarlet, and the narrow absorption band at about 615 mμ. of alkaline protohaematin to be replaced by a broad, somewhat diffuse band in the green region of the spectrum at 545 mμ. According to Hogness, Zscheile, Sidwell & Barron (1937) cyanhaematin contains 2 mol. of cyanide/mol. of haematin. If, however, CH_3NC or CH_3CN is added to protohaematin in ethanolic solution, there is no change in the haematin spectrum, an indication that neither of these compounds reacts with haematin. Similar negative results were obtained with urohaematin.

Methaemoglobin. KCN also combines with acid methaemoglobin, altering the colour of the solution from reddish brown to bright red and causing the absorption band at 630 mμ. to be replaced by a broad band the centre of which lies at about 540 mμ. Neither methyl *iso*cyanide nor methyl cyanide causes any change in the colour or spectrum of methaemoglobin, and it may therefore be assumed that these compounds do not combine with it.

DISCUSSION

Haemochromogens have hitherto been defined as compounds of haem with 2 mol. of a nitrogenous base. Potassium cyanide and methyl *iso*cyanide, however, differ from all other nitrogenous substances investigated so far, in that each can form with haem two distinct compounds containing 1 and 2 mol. of the substance per molecule of haem.

In his study of the two cyanide-haem compounds, Hill (1929) found that on the addition of CO to (CN^-)-haem, ordinary CO-haem was formed and not a CO-(CN^-)-haem compound, the single cyanide ion being replaced by CO. He stated, however, that 'the existence of a compound such as CO-cyan-haemochromogen is not excluded, but so far it has not been detected'. It is now shown that there is ample spectroscopic evidence for the existence of a definite CO-(CN^-)-haem. The conditions required for its formation show that in the presence of excess cyanide, i.e. when $(CN^-)_2$-haem is formed, one of the CN^- groups of this compound can be replaced by CO giving CO-(CN^-)-haem. Apparently in (CN^-)-haem, CO can replace only the (CN^-)-group and not the water co-ordinated with the iron atom, as shown in the scheme on p. 444.

The CO-(CN^-)-haem is of special interest since it shows that two important inhibitors of respiration and of reactions catalysed by certain haematin compounds may, under certain conditions, co-ordinate simultaneously with the same iron atom of haem.

A comparative study of the properties of the two cyanide-protohaem compounds and ordinary haemochromogens (e.g. globin or pyridine haemochromogen) shows that: (1) (CN^-)-haem has an absorption spectrum of the same pattern as that of a haemochromogen but, unlike the latter, it contains only 1 mol. of nitrogenous base/atom of Fe; its absorption bands both in the visible and violet regions of the spectrum are considerably lower than those of a haemochromogen, and it does not form a CO compound of the CO-haemochromogen type. Unfortunately, its magnetic susceptibility has not yet been determined, for it would be very interesting to know whether or not the compound is diamagnetic as suggested by its absorption spectrum. (2) $(CN^-)_2$-haem resembles a haemochromogen in its composition (2 mol. of nitrogenous substance per atom of Fe), in the zero magnetic moment of the Fe atom (Pauling & Coryell, 1936) and in forming a CO-(CN^-)-haem compound analogous to a CO-haemochromogen, but it differs from a haemochromogen in several ways. The patterns of the absorption spectra of $(CN^-)_2$-haem and its CO derivatives are entirely different from those of a haemochromogen and CO-haemochromogen respectively and, in addition, the densities of the absorption bands of $(CN^-)_2$-haem are only about half of those of a typical haemochromogen.

Methyl *iso*cyanide has now been shown to give two compounds with haem analogous to those given by KCN. Since methyl *iso*cyanide (CH_3NC) is only able to combine with the haem Fe by means of its terminal carbon atom, it may be assumed that in both mono- and di-cyan-haem the cyanide ion is also linked by its carbon atom. Additional evidence in favour of this view is provided by the inability of methyl cyanide (CH_3CN) to react with haem either in the manner of KCN or as a simple haemochromogen-forming base in spite of its terminal nitrogen atom. These results corroborate the views of Pauling & Coryell (1936) and Wyman (1948) that 'in the dicyanide compound it is probably the carbon atoms of the cyanide ions which are linked with the iron'. It is this mode of linkage which is probably responsible for the fundamental differences between the cyanide and carbylamine haem compounds on the one hand and the true haemochromogens on the other. In the latter type of compound the nitrogenous base is always linked to the haem Fe through the nitrogen atom.

It is of interest to note that methyl *iso*cyanide reacts with the bivalent iron of haem and haemoglobin but not with the tervalent iron of haematin and methaemoglobin. In this respect methyl *iso*cyanide resembles CO, with which it can compete for ferrous haem and haemoglobin.

The structure of dicyan-haem is of particular interest since the pattern of its absorption spectrum bears a strong resemblance to a certain haem derivative which was first described by Dhéré & Vegezzi (1916) and which will be discussed in the next paper.

SUMMARY

1. As was previously shown, potassium cyanide gives rise to two distinct compounds with haem: monocyan-haem (1 CN^-/Fe) and dicyan-haem (2 CN^-/Fe).

2. The absorption curves of these compounds have been determined in the visible as well as the violet regions of the spectrum and the marked differences in their pattern are discussed in relation to the spectra of other haematin derivatives.

3. Methyl *iso*cyanide (CH_3NC) combines with haems to give mono- and di-carbylamine-haem compounds having the same spectroscopic properties as mono- and di-cyan-haems, whereas methyl cyanide (CH_3CN) does not combine with haems. These results show that the cyanide ions in mono- and di-cyan-haems are attached to the haem iron through their carbon atoms. This mode of attachment accounts for the difference between the absorption spectra of $(CN^-)_2$-haem or $(CH_3NC)_2$-haem and those of ordinary haemochromogen compounds such as pyridine haemochromogen.

4. It is only when one cyanide ion or one molecule of methyl *iso*cyanide is attached to the haem iron through its carbon atom that the absorption spectrum of the resulting compound resembles that of an ordinary haemochromogen.

5. Methyl *iso*cyanide, like carbon monoxide, combines only with haem and haemoglobin and does not react with the ferric compounds haematin and methaemoglobin, both of which form characteristic compounds with cyanide ion.

6. Methyl cyanide was found not to react with any of the haematin compounds examined.

7. The existence of a CO-(CN^-)-haem compound in which iron is simultaneously co-ordinated with cyanide and carbon monoxide has been demonstrated, and an analogous CO-(CH_3NC)-haem compound is also described.

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On the Properties and Nature of Dihydroxyl-haem

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Dhéré & Vegezzi (1916), while working on the reduction of haematin under different conditions, described a compound obtained by reducing an ethanolic solution of haematin in strong alkali with sodium dithionite ($Na_2S_2O_4$) in air. This compound, which they named 'alkaline haemochromogen', was red in colour and showed a characteristic absorption spectrum consisting of two bands, of which the β-band lying nearer the blue end of the spectrum was much stronger than the α-band. It was not until several years later, however, that the term 'haemochromogen' was defined by Anson & Mirsky (1925) as covering the compounds of reduced haematin or haem with different nitrogenous substances, and Hill (1926) showed conclusively that in a haemochromogen 2 mol. of an organic base are combined with the one iron atom of haem. Of all the nitrogenous compounds examined only potassium cyanide and methyl *iso*cyanide (methyl carbylamine) were found to form two distinct compounds with haem: monocyan-haem and dicyan-haem, which contain 1 and 2 mol. of cyanide per molecule of haem respectively, and mono- and di-carbylamine haem (see Keilin, 1949).

The object of this investigation is to elucidate the nature of the compound described by Dhéré & Vegezzi as 'alkaline haemochromogen' and to compare it with haem, monocyan-haem, dicyan-haem and haemochromogens. It is proposed to describe the 'alkaline haemochromogen' of Dhéré & Vegezzi under the name of dihydroxyl-haem, with a prefix to the haem denoting the type of porphyrin used; e.g. dihydroxyl-protohaem and dihydroxyl-urohaem.

MATERIAL AND METHODS

Protohaemin. This was prepared by the method of Schalfejeff (1885) from ox or horse blood.

Haematohaemin. Haematoporphyrin was prepared from protohaemin by Nencki's method (Nencki & Seiber, 1888) and the iron was introduced by treating the porphyrin with $FeSO_4$ in the presence of glacial acetic acid and sodium acetate.

Urohaemin. Uroporphyrin I was isolated from the urine of a case of congenital porphyria. The porphyrin was esterified and the urohaemin prepared from the octamethyl ester according to Fischer & Orth (1934).

Spectroscopic observations. For all qualitative spectroscopic examinations and for preliminary quantitative experiments a microspectroscope was used as previously described (Keilin, 1943). The recording of absorption spectra in the visible and violet regions was carried out with a Beckman photoelectric spectrophotometer. In all figures the molecular absorption coefficients (ϵ), as previously defined (Keilin, 1949), were plotted against wavelengths (in mμ.).

RESULTS

The reaction of protohaem with sodium hydroxide

A solution of protohaem, obtained by dissolving protohaemin in 0·1 N-NaOH or 1% (w/v) Na_2CO_3 (anhydrous) and reducing it with $Na_2S_2O_4$, shows two very diffuse absorption bands. The addition of ethanol to this solution affects this spectrum only in so far as it causes a very slight sharpening of the bands. If, however, the ethanolic solution of protohaematin contains a much higher concentration of NaOH, the solution becomes cherry coloured on reduction, and a characteristic two-banded spectrum is seen in which the β-band, with its centre at