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REACTIONS OF CYANAMIDE WITH METHAEMOGLOBIN AND SOME OTHER HAEMATIN COMPOUNDS

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SUMMARY

Cyanamide reacts with methaemoglobin to give a red compound with a characteristic absorption spectrum. Cyanamide also combines with whale metmyoglobin and Tubifex methaemoglobin. These compounds are best formed in alkaline solution, one molecule of cyanamide combining per atom of haematin iron. Cyanamide acts as an inhibitor of the cytochrome oxidase system but is 300 times less effective than cyanide or azide. Contrary to certain claims, no compound formation was observed between cyanamide and free haematins, haems or their derivatives. The significance of these results and the possible mode of combination of cyanamide with methaemoglobin are discussed in the light of the effects of KCN, methyl isocyanide and acetonitrile on haemoproteins and free haematins.

INTRODUCTION

It was shown by BARNARD[1] that cyanamide gives with methaemoglobin a compound which is well defined spectroscopically. The pharmacological properties of cyanamide are of particular interest in that they differ very markedly from those of potassium cyanide; the characteristic effect of cyanamide intoxication in subjects exposed to it, either in industry or when using it as calcium cyanamide for agricultural purposes, consisting of the potentiation of the vasodilator effect of alcohol on the circulatory system[1].

It was of interest to reinvestigate quantitatively the reactions of cyanamide with haematins and haemoproteins in order to compare them with those given by potassium cyanide, methyl isocyanide, acetonitrile[4], azide and some other nitrogenous substances.

MATERIALS AND METHODS

Cyanamide

This is a very unstable substance which rapidly polymerises to dicyanamide. Pure cyanamide, kindly supplied by Dr. HACKMAN, was stored in several small containers in a desiccator (non-evacuated) over sulphuric acid. Under these conditions polymerisation was largely prevented and samples stored in this way for over a year still give a dark purple colour when added to potassium ferrocyanide and exposed to

light. This colour reaction[3] is specific for cyanamide and is not given by dicyanamide, urea or cyanide.

Methyl cyanide (acetonitrile)

The laboratory grade (B.D.H.) was purified of free cyanide and isocyanide by adapting the method of TODA[4] for the purification of butyl cyanide.

Horse methaemoglobin

This methaemoglobin was prepared from crystalline oxyhaemoglobin by oxidising it with a small amount of $K_3Fe(CN)_6$ and dialysing against several changes of water. The concentration of the solution was determined as cyanmethaemoglobin[5].

Tubifex methaemoglobin

Fresh, live *Tubifex tubifex* (fresh-water oligochaet worms) were homogenised with water; the fluid was centrifuged, the fatty layer and precipitate were discarded and the red supernatant was fractionated with $(NH_4)_2SO_4$. The fraction precipitating between 52 and 60% saturation of $(NH_4)_2SO_4$ was dialysed against water till salt-free. The precipitated haemoglobin was redissolved by adding a few millilitres of 0.1 M phosphate buffer (pH 6.0), and oxidised with $K_3Fe(CN)_6$. The methaemoglobin was then dialysed against several changes of water and finally against 0.033 M phosphate buffer (pH 6.0).

Sperm whale metmyoglobin

This was kindly supplied by Dr. J. C. KENDREW.

Catalase

This was prepared from horse liver as described by KEILIN AND HARTREE[6].

Cytochrome oxidase

A preparation following the method of SMITH AND STOTZ[7] was used and its activity was estimated spectrophotometrically as described by SMITH[8].

Cytochrome c

This substance was purified from horse-heart muscle by the method of KEILIN AND HARTREE[9] and chromatographed on Amberlite IRC-50 as described by MARGOLIASH[10]. Cytochrome *c* in 0.1 M phosphate buffer (pH 7.4) containing 2.5 mM sodium versenate, was reduced with ascorbic acid and dialysed overnight against the same versene–buffer mixture. After suitable dilution with 0.1 M phosphate buffer (pH 7), the concentration was determined spectrophotometrically assuming $\varepsilon = 2.95 \cdot 10^4$ as recommended by VAN GELDER AND SLATER[11].

Haemins

Protohaemin was prepared from horse or pig blood by the method of SCHALFEJEFF[12]. Uroporphyrin I was isolated from the urine of a case of congenital porphyria and crystallised as the ester. Urohaemin was then prepared from the uroporphyrin ester[13].

Spectroscopic methods

A Beck microspectroscope was used for the direct observation of the absorption spectra in the visible region. Spectrophotometric curves were determined with a Unicam SP 500 spectrophotometer using 1.0- or 0.5-cm cells.

RESULTS

Reactions of cyanamide with methaemoglobin

As is well known, methaemoglobin presents two different forms of absorption spectra depending on the pH of the solution. Below pH 6.8 the brown, acid methaemoglobin shows a pronounced absorption band at 635 mμ and gradually increasing absorption to a maximum at 500 mμ in the visible region, while at about pH 9.5 the solution is reddish-brown in colour, and has two bands in the green region, the α-band at 578 mμ having a well marked shoulder centred at about 605 mμ while the stronger β-band lies at 542 mμ.

On the addition of excess cyanamide to a solution of methaemoglobin in 0.1 M phosphate buffer (pH 7.6), the brown solution becomes scarlet and direct observation with the microspectroscope shows that the absorption band at about 630 mμ becomes very much weaker while two new bands appear at 574 and 540 mμ giving a spectral pattern completely unlike that of cyanmethaemoglobin. The bands at 574 and 540 mμ correspond to those described by BARNARD[1] as lying at about 579.5 and 537.5 mμ, respectively. At more alkaline pH values when the cyanamidemethaemoglobin compound is fully formed, its absorption spectrum closely resembles that of azidemethaemoglobin in both the positions and relative intensities of the absorption bands in the visible region, the similarity being less marked in the Soret bands (Figs. 1a and 1b).

In more acid solutions however, (pH 6.0), cyanamide hardly combines with methaemoglobin and only causes a slight intensification of its absorption spectrum at about 575 and 540 mμ, whereas azidemethaemoglobin is fully formed under these conditions (Fig. 1c). Acidification to pH 6.0 of fully formed cyanamidemethaemoglobin in alkaline solution in the presence of excess cyanamide, causes a progressive change in both colour and absorption spectrum to that of free acid methaemoglobin; this change is completely reversible. The effect of pH on the positions and extinction coefficients of the Soret band of methaemoglobin and its cyanamide and azide compounds are compared in Table I.

The affinity of horse methaemoglobin for cyanamide was determined at various pH values as follows. A series of twelve tubes was prepared, each containing 0.25 ml of methaemoglobin ($4.5 \cdot 10^{-4}$ M in terms of its haematin content) and 0.5 ml of 0.4 M buffer (phosphate at pH 6.05 and 6.99, borate at pH 8.0 and 9.0 and carbonate–bicarbonate at pH 10.0). To the first tube water was added to a total volume of 1.5 ml while to the other tubes were added different amounts of a freshly prepared solution of 1 M or 2 M cyanamide, the total volume in each tube being made up to 1.5 ml with water. The extinction was measured spectrophotometrically in 0.5-cm cells at 574 and 540 mμ, the peaks of the α- and β-bands respectively of the cyanamide-methaemoglobin compound. The extinction at infinite concentration of cyanamide at each pH was obtained as described in the study of the phenol–methaemoglobin compound by GEORGE, LYSTER AND BEETLESTONE[14]. In this method, a plot is made

of $a/[x]$ against A (absorbancy at one particular wavelength) where $a = A - A_0$, A_0 being the initial absorbancy and $[x]$ being the concentration of cyanamide. The line through the experimental values gives $A\infty$ at the intercept where $a/[x] = 0$. The other intercept, where $A = 0$ may be calculated from $A \times$ slope of the line, and gives

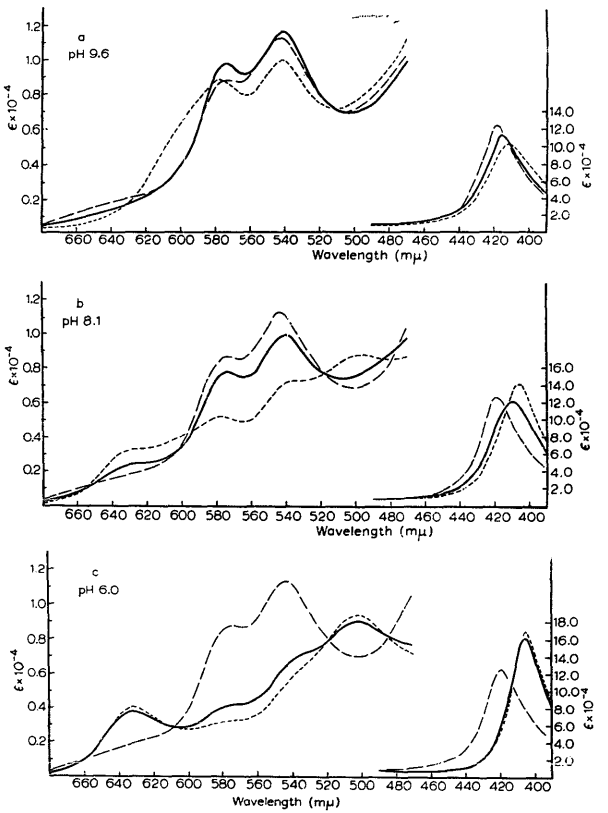

Fig. 1. Effect of pH on the formation of the cyanamidemethaemoglobin compound and azidemethaemoglobin. Absolute absorption spectra of horse methaemoglobin (– – – –) and its cyanamide (———) and azide (— —) compounds in 0.1 M $NaHCO_3$–Na_2CO_3 buffer (pH 9.6), 0.1 M borate (pH 8.1) and 0.1 M phosphate buffer (pH 6.0); cyanamide and azide added in solid form to spectrophotometer cell.

the value for A_∞/K where K is the dissociation constant of the compound. The average values for K, calculated from data measured at each of the two wavelengths, were plotted against pH (Fig. 2) and show that the greatest affinity of horse methaemoglobin for cyanamide is at pH 9 where $K = 6.65 \cdot 10^{-2}$ at 20°. It was calculated that under these conditions the stoicheiometric relationship was 1 molecule of cyanamide per 1 atom of haematin iron.

For comparative purposes horse methaemoglobin was titrated with sodium

TABLE I

POSITIONS AND EXTINCTION COEFFICIENTS OF THE SORET BANDS OF METHAEMOGLOBIN AND ITS CYANAMIDE AND AZIDE COMPOUNDS AT VARIOUS pH VALUES

Buffers used: 0.1 M phosphate for pH 6.0–7.6; borate for pH 8.1–8.9; $NaHCO_3$–Na_2CO_3 for pH 9.6 and 10.0.

pH	*Methaemoglobin*		*Cyanamidemethaemoglobin*		*Azidemethaemoglobin*	
	mμ	ε × 10⁻⁴	mμ	ε × 10⁻⁴	mμ	ε × 10⁻⁴
6.0	405	16.8	405	16.2	418	12.7
7.0	405	16.08	406	14.9	—	—
7.6	406	15.44	407	12.95	418	12.64
8.1	406	14.2	409.5	12.2	418	12.64
8.5	406	12.48	412.5	11.45	418	12.59
8.9	409	11.23	415	11.5	418	12.36
9.6	412	10.2	415	11.28	418	12.25
10.0	412	9.04	416	11.06	—	—

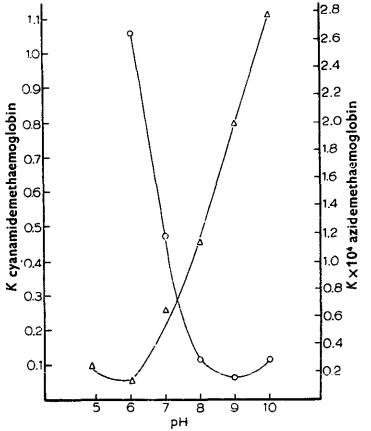

Fig. 2. Effect of pH on the dissociation constant (K) of cyanamide- and azidemethaemoglobin. Horse methaemoglobin ($4.5 \cdot 10^{-4}$ M in terms of haematin) titrated with 1 and 2 M cyanamide and with $1 \cdot 10^{-3}$ and $2 \cdot 10^{-3}$ M azide. Buffers, overall concentration in reaction mixture 0.1 M: acetate (pH 4.8), phosphate (pH 6.05, 6.99), borate (pH 8.0, 9.0), carbonate–bicarbonate (pH 10.0). K calculated as described in text.

azide (NaN_3). The methaemoglobin and buffer concentrations in each tube were the same as described above and varying amounts of a $1.0 \cdot 10^{-3}$ M solution of sodium azide were added. The extinction of each solution was read at 572 and 542 mμ, the peaks of the absorption bands of the azidemethaemoglobin compound. The results, which are also given in Fig. 2, show that the greatest affinity of horse methaemoglobin for azide is at pH 6.0 when $K = 1.49 \cdot 10^{-5}$ at 20°, that is, about 4000 times greater than the optimum affinity of methaemoglobin for cyanamide.

Effect of low temperature on cyanamidemethaemoglobin

It is generally accepted that the 6th coordination place of the iron in acid methaemoglobin is occupied by a molecule of water and that the formation of alkaline methaemoglobin involves the loss of a proton[15]. When alkaline methaemoglobin is cooled in liquid air, it reversibly changes both its colour and absorption spectrum to that of the acid form[16], these changes being due to the suppression of the ionisation at very low temperatures.

As the absorption spectrum of cyanamidemethaemoglobin also bears some resemblance to that of alkaline methaemoglobin, it was important to compare the effect of low temperature on both compounds.

When cyanamidemethaemoglobin at pH 9.6 was frozen to −72° in a CO_2–acetone mixture, the colour of the frozen solution remained red and retained its characteristic absorption bands, whereas the control methaemoglobin at the same pH turned from reddish to brown and showed the spectrum of acid methaemoglobin. These results indicate that there is definite compound formation between cyanamide and methaemoglobin, the cyanamide molecule replacing the hydroxyl group coordinated with the haematin iron in alkaline methaemoglobin.

Effect of imidazole on cyanamidemethaemoglobin

In methaemoglobin the molecule of water or hydroxyl group in position 6 can be replaced by a number of ligands each giving rise to a compound with a characteristic absorption spectrum. Imidazole combines very readily with methaemoglobin giving a parahaematin type of spectrum. By adding excess cyanamide under alkaline conditions, the imidazole is displaced and the absorption bands of the cyanamidemethaemoglobin compound are seen. Similarly cyanamide may be displaced from methaemoglobin by excess imidazole or another ligand combining under the same conditions.

Effect of cyanamide on other haemoproteins

Sperm whale metmyoglobin: The reaction of cyanamide with whale metmyoglobin differed from its reaction with horse methaemoglobin only in that at pH 6.0 the cyanamidemetmyoglobin compound was more clearly detectable than the cyanamidemethaemoglobin compound. However, the properties of these two compounds when fully formed were very similar.

Tubifex methaemoglobin: The haemoglobin of Tubifex has a mol. wt. of about $3 \cdot 10^6$ and an equivalent weight per haem of 50000 (see ref. 17). Tubifex methaemoglobin resembles horse methaemoglobin in its acid and alkaline forms and in its reactions with ligands such as fluoride, cyanide, azide and imidazole[18] but the positions of the absorption bands of Tubifex methaemoglobin and its derivatives lie 3–4 mμ

nearer the blue end of the spectrum than the bands of the corresponding compounds of horse methaemoglobin.

With cyanamide, Tubifex methaemoglobin gave a compound very similar to that given by horse methaemoglobin but the reaction took place over a much wider pH range. Thus at pH 6, the reaction was almost complete and only a trace of the 624 mμ band of acid methaemoglobin remained visible; at pH 5.2 it was slightly less well formed while at pH 4.8 the characteristic pattern of the cyanamide compound was still perceptible although under these conditions the solution became opalescent.

Other forms of haemoglobin: No reaction was found between cyanamide and oxyhaemoglobin, haemoglobin, COhaemoglobin or denatured globin haemochromogen showing that cyanamide does not combine with ferrous derivatives of haemoglobin.

Catalase: In the case of catalase, the addition of cyanamide did not affect the colour of the solution and caused only a minor change in the absorption spectrum. This, as seen in Fig. 3, consists of the appearance of a shoulder centred at about 585 mμ, the rest of the spectrum including the Soret band remaining unchanged. The problem of the significance of this change in the absorption spectrum and whether it may indicate a reaction between cyanamide and the prosthetic group of catalase, can only be settled by further investigations.

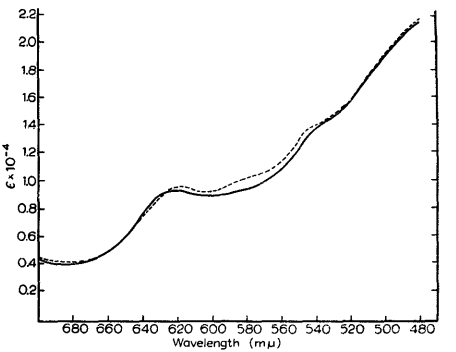

Fig. 3. ———, absolute absorption spectrum of horse liver catalase in 0.26 M phosphate buffer (pH 7.6); – – – –, effect of cyanamide.

Peroxidase: No reaction could be detected between cyanamide and highly purified horse-radish peroxidase.

Cytochrome c: No change was observed with the microspectroscope in the absorption bands of oxidised or reduced cytochrome *c* in the presence of cyanamide.

Effect of cyanamide on free haematins and haems

It had been claimed by BARNARD[1] that cyanamide is capable of combining with free haematin to form a ferrihaemochromogen (parahaematin) and that cyanamide also

reacts with ferrihaemochromogen compounds to give a distinctive coloured product which may form the basis of a colour test for cyanamides, the example given being the purple colour developed after the addition of cyanamide to a solution of haematin in 0.1 % (w/v) buffered cetylpyridinium chloride, which he referred to as cetylpyridinium ferrihaemochromogen[1].

In the present study, however, it was found that cetylpyridinium chloride does not combine with haematin to give a parahaematin but merely has a solubilising effect similar to that of alcohol[19], as will be described elsewhere.

The term parahaematin as used here, refers to a compound of haematin in which the 5th and 6th coordination places are both occupied by molecules of a nitrogenous substance such as imidazole or pyridine, which combine with the iron through nitrogen atoms. Cyanide (KCN), on the other hand, combines readily with haematins giving the stable, red, cyanhaematin in which the cyanide groups are most probably linked to the haematin iron through their carbon atoms.

As cyanamide possesses both an amide and a cyanide group and in view of BARNARD's claims[1], it was of interest to examine its action on haematins and haems and to compare it with the reactions previously described[2] with cyanide (KCN), methylisocyanide (CH_3NC) or acetonitrile (CH_3CN). Urohaemin was used for these investigations in addition to protohaemin because, owing to its eight carboxyl side chains, it is much more soluble and was found to react with many substances such as amino acids with greater ease than protohaemin[18].

The effect of cyanamide on alkaline proto- and urohaematins and haems was examined at various pH's and concentrations and in the presence and absence of dispersing agents such as alcohol and caffeine, but no changes indicating compound formation with these haematins or haems were observed. Neither did cyanamide have any effect on the colour or spectrum of imidazoleparahaematin.

In view of these findings, the effects of pure methylcyanide (acetonitrile) on methaemoglobin, haematins and haems were re-investigated under a wider range of conditions than before. No compound formation between these and methylcyanide was found, thus confirming previous observations[2]. With free protohaematin, however, there were minor changes in the absorption spectrum when excess methylcyanide was added, but these were most probably due to alterations in the degree of dispersion of the pigment such as can be produced by the addition of ethanol or acetone.

Action of cyanamide on the cytochrome oxidase system

It was stated by BARNARD[1] that cyanamide can act as "a tissue asphyxiant" and that in this respect it has a much lower toxicity than cyanide. Since no experimental data were presented in support of this statement and no other investigations appear to have been carried out on this problem since 1944, it was decided to do a preliminary investigation on the effect of cyanamide on the activity of the cytochrome oxidase system using a heart-muscle preparation very rich in cytochromes *a* and a_3. The effect of cyanamide on this system was compared with that of azide, as both these substances gave spectroscopically similar compounds with methaemoglobin.

0.5 ml of a $1.28 \cdot 10^{-4}$ M solution of reduced cytochrome *c* was placed in a 1.0-cm spectrophotometer cell together with 2.2 ml of 0.1 M phosphate buffer (pH 7.0). The extinction was measured against a control cell containing water. At zero time 0.1 ml of the cytochrome oxidase preparation was pipetted into the solution, stirred

and the change in extinction was read against time, the concentration of the oxidase having been adjusted so as to give almost complete oxidation in about 4–5 min. One drop of a saturated solution of potassium ferricyanide was then added to complete the oxidation and the extinction was read again. Where inhibitors were used, the total volume of the reaction mixture was adjusted to 2.8 ml by varying the amount of buffer. The ranges of concentration of the inhibitors were $9 \cdot 10^{-4}$ to 0.1 M cyanamide and $7 \cdot 10^{-5}$ to $7 \cdot 10^{-4}$ M azide. The result of this experiment showed that to obtain 50 % inhibition of cytochrome oxidase activity, the concentration of cyanamide must be about 300 times greater than that of azide.

DISCUSSION

In the present study of the reactions of cyanamide with free haemoproteins, haematins and their derivatives, it was found that cyanamide combined only with methaemoglobin and metmyoglobin, giving a red compound with two well-defined absorption bands in the green region of the spectrum. Cyanamide did not combine with any ferrous derivative of haemoglobin as previously reported[1], nor with cytochrome *c* in either the ferrous or ferric forms. With horse-liver catalase, cyanamide did produce a slight change in the absorption spectrum but the significance of this change is doubtful.

The action of cyanamide as a "tissue asphyxiant" of much lower toxicity than cyanide, as described by BARNARD[1], was confirmed by studies of its effect on the cytochrome oxidase system. Cyanamide was found to be very much less effective as an inhibitor than azide which, in this system, has an inhibitory action of the same order of magnitude as cyanide[20].

The formation of cyanamidemethaemoglobin is pH-dependent, but whereas with horse methaemoglobin the compound was formed only in alkaline solution (Fig. 1) with sperm whale myoglobin it was already partially formed at pH 6. Tubifex methaemoglobin which is an acidic haemoprotein with an isoelectric point[21] at pH 5.8, combined with cyanamide between pH 4.8 and 10, the compound being already almost completely formed at pH 6. In this connection it was shown by SCHELER[18] that there are close analogies between Tubifex and vertebrate methaemoglobins in their combi-

TABLE II

REACTIONS OF FREE Fe–PORPHYRINS AND HAEMOPROTEINS WITH CYANIDES, CYANAMIDE AND AZIDE

	Fe^{3+}		Fe^{2+}	
	Haematin	*Methaemoglobin Metmyoglobin*	*Haem*	*Haemoglobin Myoglobin*
KCN	+	+	+*	+
CH_3NC	o	o	+*	+
CH_3CN	o	o	o	o
NH_2CN	o	+	o	o
NaN_3	+	+	o	o

* Two compounds given in each case: CNhaem and $(CN)_2$haem; CH_3NChaem and $(CH_3NC)_2$-haem.

nation with ligands, the pK of the Tubifex methaemoglobin compound being lower than the pK of the corresponding horse methaemoglobin compound.

The absorption spectrum of cyanamidemethaemoglobin (horse), most closely resembles that of azidemethaemoglobin, but whereas azidemethaemoglobin is best formed in acid solutions, optimum formation of cyanamidemethaemoglobin is at pH 9 (Fig. 2). Furthermore, the affinity of methaemoglobin for cyanamide is about 4000 times lower than for azide under optimal conditions.

The effect of low temperatures on cyanamidemethaemoglobin and the reversible replacement of cyanamide by imidazole in the compound, indicate that the cyanamide is coordinated directly with the haematin iron, one molecule of cyanamide combining per atom of iron.

The failure of cyanamide to combine with free haematins and haems is of considerable interest, since by virtue of its amide group it might theoretically have given rise to a parahaematin and haemochromogen respectively or else, by its cyanide radical, have given compounds analogous to those formed by potassium cyanide and, in the case of haem and haemoglobin, also by methylisocyanide (CH_3NC) (Table II). Although we have no direct evidence for the mode of attachment of the cyanide radical to the ferric iron of haematin or methaemoglobin, the fact that the absorption spectra of cyanhaematin and cyanmethaemoglobin are virtually the same and do not resemble the parahaematin spectrum, suggests that here, too, the cyanide radical is combined through the carbon atom.

In the case of cyanamidemethaemoglobin, not only does this compound differ from cyanmethaemoglobin in its absorption spectrum and conditions of formation, but the fact that the molecular structure of cyanamide precludes its carbon atom from reacting with the haematin iron leads us to conclude that cyanamidemethaemoglobin cannot be considered as "entirely chemically analogous" to cyanmethaemoglobin as had been suggested by BARNARD[1]. Furthermore, since methylcyanide appears unable to combine with haem or haematin, whether free or as the prosthetic group of a haemoprotein, it is unlikely, though not impossible, that cyanamide combines by the nitrogen of its cyanide radical. These facts suggest that the most probable combination of cyanamide with methaemoglobin is through its amide group. The failure of this amide group to give a parahaematin or haemochromogen presents a similar problem to that of urea which also does not give these compounds with haematin or haem for reasons as yet not understood.

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