CYANATE-METHAEMOGLOBIN

by

ROSE BADER, P. DIRNHUBER AND F. SCHÜTZ

Department of Pharmacology, The Medical School, University of Birmingham (England)

In the course of investigations[1] regarding the actions, and possible formation in the mammalian organism of the isomer of urea, ammonium cyanate, use was made occasionally[2, 3] of the reaction of cyanate (CNO') with methaemoglobin (met-Hb)[4, 5*]. This paper describes a number of new spectroscopic and chemical characteristics of the compound.

METHODS AND MATERIALS

Methaemoglobin. Human erythrocytes from citrated blood were washed 3–4 times with 0.9% NaCl. The white cells were removed as thoroughly as possible. The washed red cells were lysed by addition of 7–8 vol. of dist. water. A sufficient amount of a freshly prepared ½-saturated solution of $K_3Fe(CN)_6$ was added drop by drop, while the solution was being kept agitated. The chocolate brown liquid was left standing 1 h at room temperature, and was dialysed through cellophane or parchment against distilled water at 4° for at least 24 h. The distilled water was changed at least 12 times. 20% (v/v) of 0.2 *M*-phosphate buffer solution (if not otherwise mentioned $p_H = 7.4$) was then added, and the solution centrifuged and filtered. When not used at once the solution was kept at 4°. No solution older than 36 h was used.

Crystalline met-Hb was made according to WARBURG[6].

Methaemoglobin in intact erythrocytes. Human red cells were washed 3–4 times in a solution being 0.2 *M* with regard to phosphate buffer (p_H 7.4), and 0.14 *M* with regard to NaCl. The washed cells were suspended in the same solution and made up to the original volume. Sodium or amyl nitrite was then added while the suspension was gently stirred by a motor. After being kept agitated for 1 h, *circa* 80% of the haemoglobin present in the cells was converted into met-Hb. $K_3Fe(CN)_6$ was found less suitable than the nitrites, for this purpose. The cells were then again washed thrice in the buffered NaCl-solution, to remove excess of nitrite. The band in the red (6325 Å), characteristic for the met-Hb present in the cells, can easily be observed by means of a HARTRIDGE reversion spectroscope, using a strong light source, and agitating the suspension before and during the observation.

Stokes reagent was prepared by mixing equal volumes of 2% ferrous sulphate and 3% tartaric acid. NH_4OH was then added until the precipitate had entirely dissolved. Only the freshly made reagent was used.

Potassium ferricyanide. A commercial sample was twice recrystallized from water.

Ammonium carbamate (*B.D.H.*), *amyl* and *sodium nitrite* were used without purification.

Sodium cyanate was prepared from urea according to BADER, DUPRÉ, AND SCHÜTZ[7].

Spectroscopy. A BECKMAN photoelectric spectrometer (DU model) and a HARTRIDGE reversion spectroscope were used. The small, but significant shifts of absorption bands, caused by the reaction of the pigment with cyanate, are only detectable by means of small dispersion spectroscopes; most suitable among those was the HARTRIDGE reversion spectroscope. With the latter instrument all readings were made in a dark room, the iris being maximally opened. The pigments were diluted to the lowest suitable concentration, which still permitted clear readings of the band in the red.

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* Since cyanate-methaemoglobin was described by one of us[5] we discovered that HECHT[4] had earlier published some spectroscopic characteristics of the same compound. The German periodical in which the paper by HECHT appeared was not available during the war, neither was the investigation mentioned in any of the abstracts to which we had access.

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RESULTS

When sodium cyanate is added to a solution of met-Hb, while the band in the red at 6325 Å is being observed, the latter can be seen to shift, within 1–2 seconds, towards the smaller wavelengths. When sufficient cyanate has been added the band reaches the position of 6285 Å, characteristic for neutral or acid cyanate-met-Hb-solution. Alkaline met-Hb is known to lose the band in the red, while on addition of cyanate the band is retained, although it is found at slightly higher wavelengths (6340 Å), than in an acid milieu.

To complete the reaction, at least 80 mols of cyanate are need per mol met-Hb (Fig. 1).

Essentially the same results were obtained with a preparation of crystalline met-Hb, dissolved in 0.1 *M*-Na_2HPO_4, and subsequently brought to p_H 7.4 by addition of KH_2PO_4.

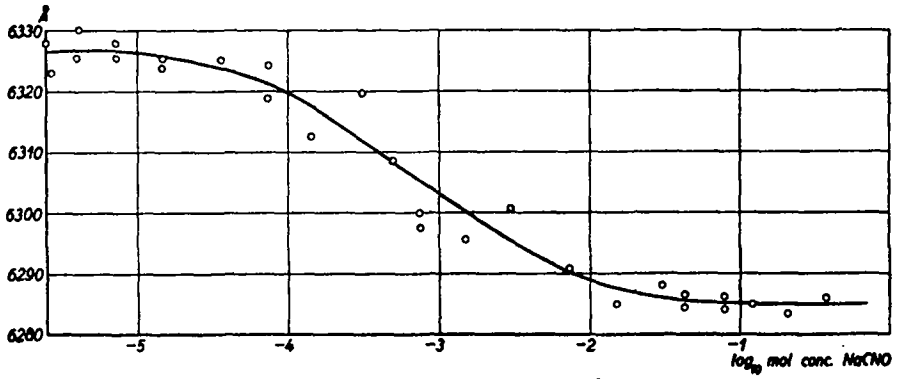

Fig. 1. The position of the absorption band in the red region (Å), determined with a HARTRIDGE reversion spectroscope. $1.88 \cdot 10^{-5}$ *M*-methaemoglobin solution in equilibrium with various concentrations of sodium cyanate

When sodium cyanate was added to suspensions in buffered NaCl solution (p_H 7.4) of human or rabbit erythrocytes, containing intracellular met-Hb, the band in the red was similarly seen to shift, as in the case of dissolved met-Hb, towards and not further than 6285 Å. The shift was regularly observed, although slightly higher concentrations of cyanate were needed to produce it. These experiments show that cyanate is capable of penetrating the red cell membranes at room temperature and p_H 7.4.

The absorption curves shown in Figs. 2–9 were all constructed by means of the BECKMAN photoelectric spectrometer. With the exception of those shown in Fig. 5 the very marked differences between the absorption curves of met-Hb, cyanate-met-Hb and thiocyanate-met-Hb have not been described so far.

The met-Hb solution, usually buffered, as mentioned in each case, was suitably diluted for each respective spectral region, and split into 3 parts. Dilute, freshly made solution of NaCNO was added to one, and equal amounts of equimolecular concentrations of KCNS and NaCl, respectively, were added to the other two parts. Care was taken that the pigment concentration remained equal in all three parts (± 0.1%). The readings were started ½–1 h after the addition to the pigment solution of the solution to be tested. The small equivalent amounts of NaCl added to the control, did not alter the absorption curves of met-Hb. The respective buffer solution, or water, containing the equimolar amount of NaCl, was used as blank.

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In Fig. 2 it can be seen that even in the ultraviolet region a significant difference exists between the 3 compounds. The very strong absorption of CNS-met-Hb in the region of 250–260 mμ, in distinction to the absorption curves of the 2 other compounds, coincides with the strong absorption of pure KCNS in the same region. The absorption curves in this region of pure aqueous solutions of NaCNO, KCNS and NaCl, with pure water as blank, are shown for comparison in Fig. 3.

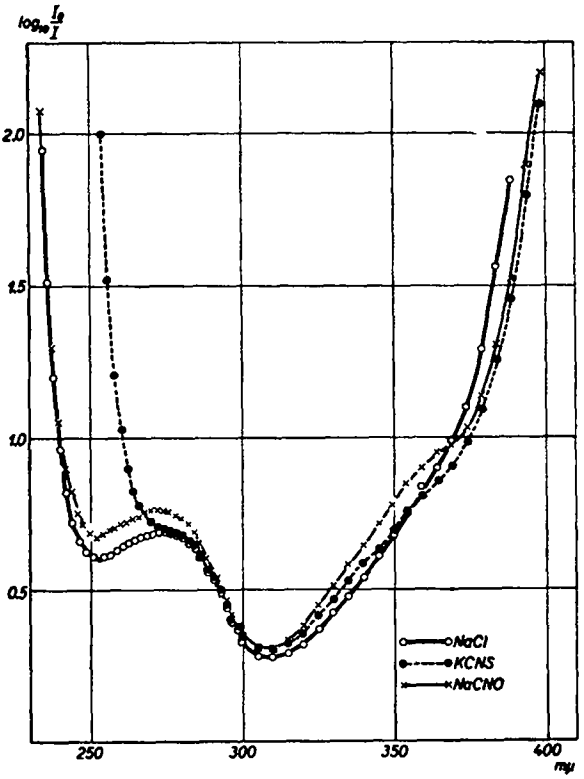

Fig. 2. Absorption spectrum (BECKMAN photoelectric spectrometer) of methaemoglobin in 1.2 *M*-phosphate buffer solution (pH 7.2), in equilibrium with 0.1 *M*-sodium cyanate (crosses), 0.1 *M*-potassium thiocyanate (full points), and, as control, 0.1 *M*-sodium chloride (circles). Blank: Solution of phosphate buffer (1.2 *M*, pH 7.2) and sodium chloride (0.1 *M*).

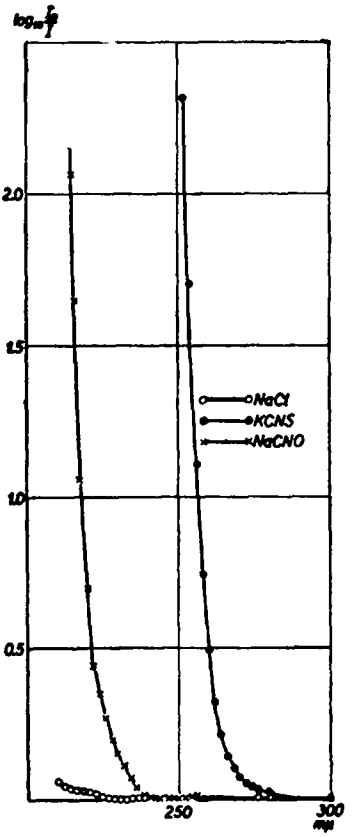

Fig. 3. Absorption spectrum (BECKMAN photoelectric spectrometer) of pure aqueous 0.1 *M* solutions of sodium cyanate (crosses), potassium thiocyanate(full points), and sodium chloride (circles). Blank: water.

In Fig. 4 it can be seen that the characteristic band in the violet-blue region (SORET band) is, through CNO′ and CNS′, decreased and shifted towards the higher wavelengths. The absorption maximum of this band is shifted from 404 mμ (met-Hb) to 411 mμ (CNO-met-Hb), and to 412 mμ (CNS-met-Hb) respectively.

The increase of absorption in the red caused by the reaction of met-Hb with CNO′, already described previously[4, 5], is again clearly evident in Fig. 5. The small and very significant shift of the band in the red, described above, however, cannot be recorded by this type of instrument (large dispersion). It should be noted that, in distinction to

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the effect on the SORET-band, in this region (6325 Å) CNO′ and CNS′ cause a shift and change in opposite directions. CNO′ increases the absorption and shifts the maximum circa 40 Å towards the smaller wavelengths (6285 Å), while CNS′ decreases the absorption and shifts the maximum towards the higher wavelengths (6400 Å).

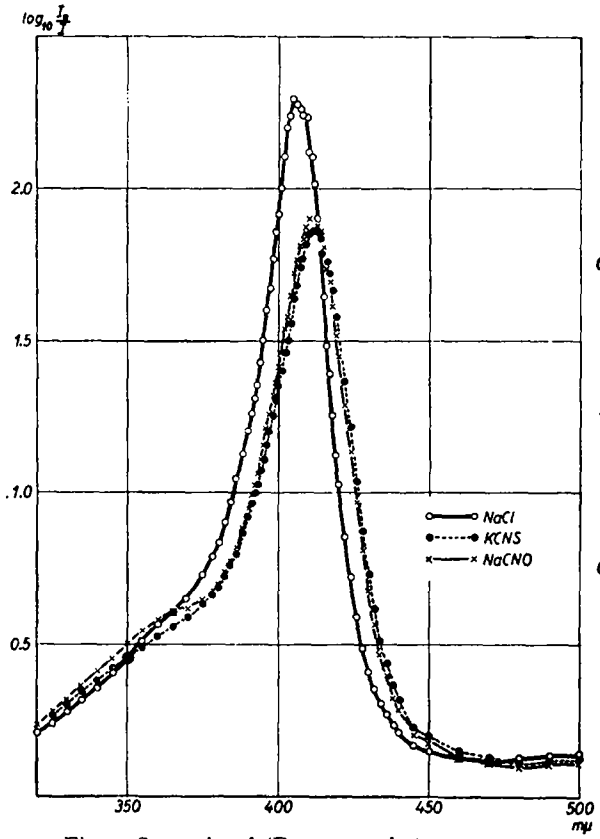

Fig. 4. SORET-band (BECKMAN photoelectric spectrometer) of methaemoglobin in 0.8 *M* phosphate buffer solution (pH 7.2), in equilibrium with 0.1 *M* solutions of sodium cyanate (crosses), potassium thiocyanate (full points), and sodium chloride (circles). Blank: Solution of phosphate buffer (0.8 *M*, pH 7.2) and sodiumchloride (0.1 *M*).

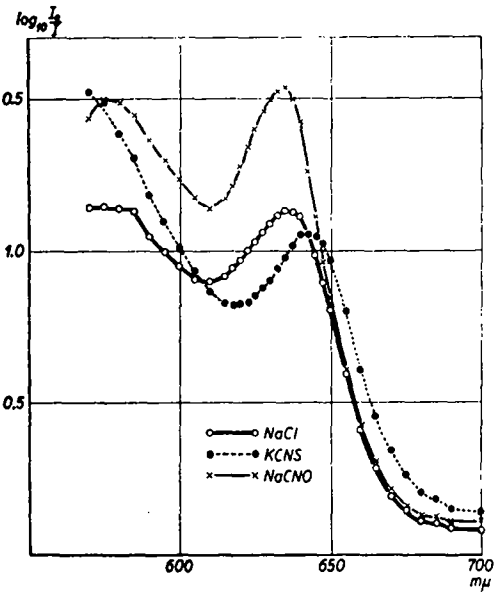

Fig. 5. Absorption spectrum of methaemoglobin (BECKMAN photoelectric spectrometer). Solutions as in Fig. 4.

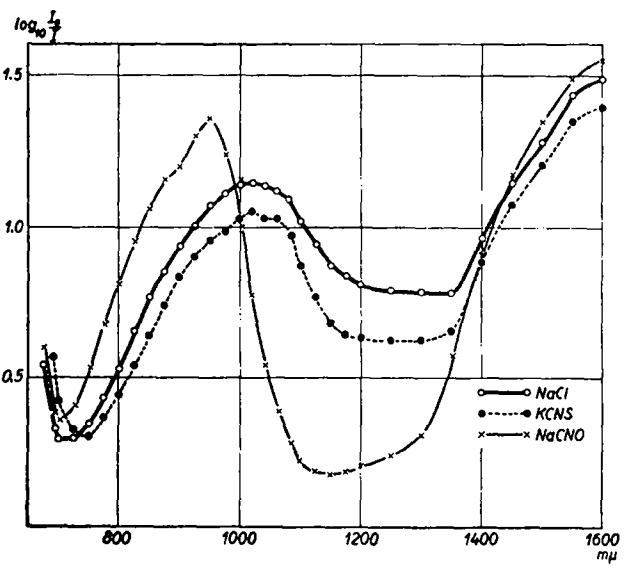

Fig. 6. Absorption spectrum (infrared region) of methaemoglobin (BECKMAN photoelectric spectrometer). Solutions as in Fig. 4.

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In Fig. 6 (infrared region) is shown what seems the most striking change of absorption caused by cyanate. The maximum of the band at 1035 mμ (met-Hb) is shifted to 950 mμ through addition of CNO′ and the absolute absorption is very much increased at the same time. CNS′, similarly to its effect on the band in the red, but in distinction to its effect on the SORET-band, here causes a small shift into the opposite direction, *i.e.*, towards higher wavelengths, and markedly decreases the absolute intensity of absorption.

A further marked difference occurs at the higher wavelengths, immediately after the band in the infrared region. Both CNO′ and CNS′ markedly decrease the absolute intensity of absorption at 1075–1125 mμ, the maximum depression of the curve being

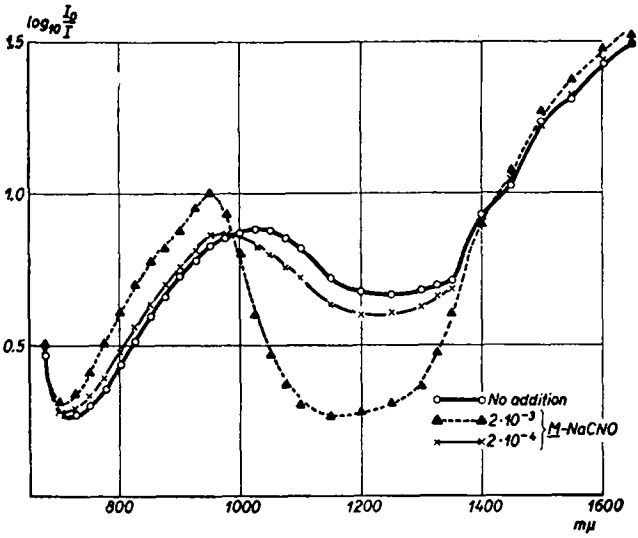

Fig. 7. Absorption spectrum in the infrared region (BECKMAN photoelectric spectrometer) of methaemoglobin in 0.08 *M*-phosphate buffer solution (pH 6.3), without any further addition (circles) in equilibrium with $2 \cdot 10^{-4}$ *M* (crosses), and $2 \cdot 10^{-3}$ *M* (triangles) sodium cyanate.

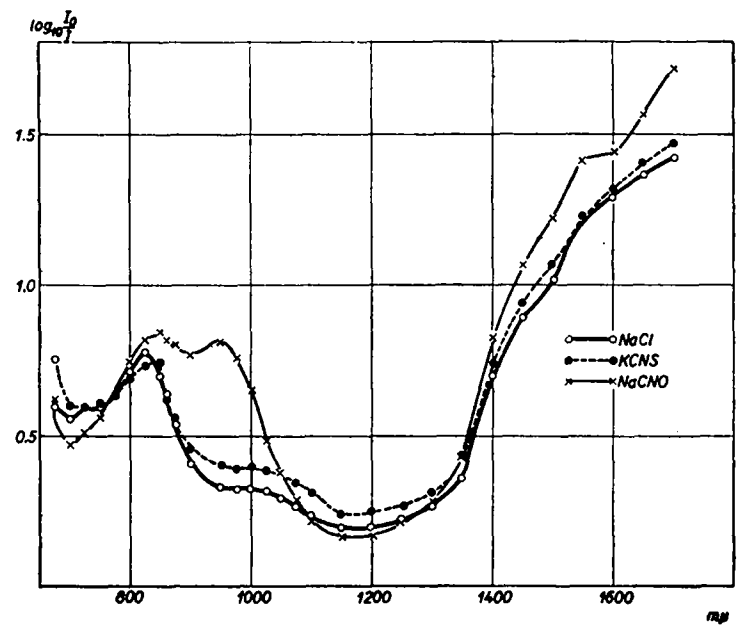

Fig. 8. Absorption spectrum (infrared region) as Fig. 6, but in alkaline milieu. 0.08 *M* phosphate buffer solution, pH 9.0. The blank consisted of this buffer solution, containing 0.1 *M* sodium chloride. Other solutions as in Figs. 6 and 4.

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situated at 1100 mμ. The decrease of absorption caused by CNO′ is far more marked than that produced by CNS′.

Of all regions of the absorption spectrum of met-Hb, the infrared region seems the most sensitive towards small additions of cyanate. In Fig. 7 it can be seen that cyanate present in $2 \cdot 10^{-4}$ *M* concentration already causes a clearly detectable change. The change in this region is more significant at neutral or slightly acid p_H than at p_H 9 (Fig. 8).

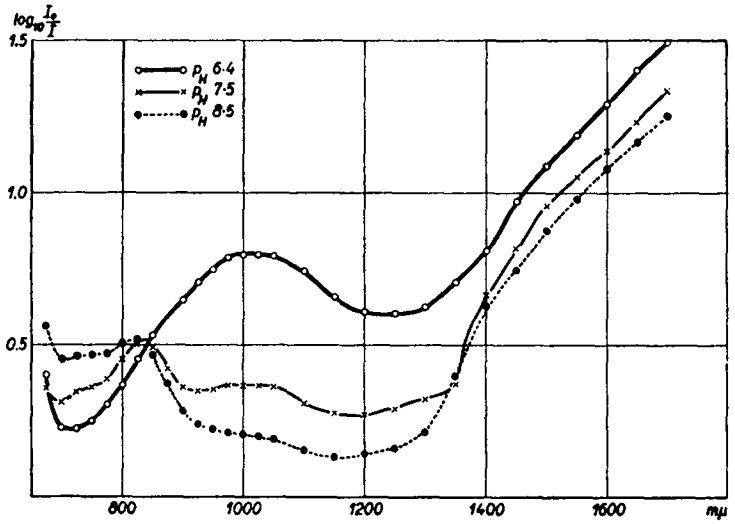

Fig. 9. Absorption spectrum (infra red region, BECKMAN photoelectric spectrometer) of pure methaemoglobin solutions, brought to different p_H. *Circles:* p_H 6.4 (natural p_H of methaemoglobin solution after dialysis). *Crosses:* p_H 7.5, *full points:* p_H 8.5. The latter p_H values were produced by addition of 0.001 *N*-NaOH. Pigment concentration was equal in all 3 solutions.

The effect shown in Figs. 6 and 7 was obvious in the presence of 0.02–0.4 *M*-phosphate buffer solution (p_H 6.2–7.2), or in the absence of buffer, when the p_H was adjusted by electrometric titration (glass electrode) to similar values, with 0.001 *N*-NaOH or NH_4OH. Fig. 9 clearly shows that the described effect could not be attributed to p_H changes; alkalinization of pure met-Hb brought about a change in the opposite direction than that caused by cyanate, which, if not buffered, would cause the p_H to shift slightly towards the alkaline side.

COMPARISON OF AFFINITIES FOR METHAEMOGLOBIN OF CYANATE AND OTHER COMPOUNDS

A solution of fluoride-haemoglobin (Hb-F) was prepared by adding a solution of sodium fluoride to met-Hb in just sufficient quantity to produce the characteristic spectral changes. When NaCNO was added to such a solution of Hb-F, even small amounts promptly produced the appearance of the band in the red, characteristic for CNO-met-Hb. When, however, NaF was added to a solution of CNO-met-Hb, *circa* 100 times the amount of NaF was needed than in the case when the NaF was added to pure met-Hb, to produce some of the spectral characteristics of Hb-F.

These experiments indicate that cyanate combines with the iron of met-Hb, and that its affinity for this iron is greater than that of fluoride. Relatively small amounts

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of CNO′ can displace fluoride from, or can inhibit the latter to combine with, the iron of met-Hb.

Unlike fluoride, both cyanide and azide showed greater affinity for met-Hb than cyanate. The latter was not capable, except in great excess, of producing a detectable amount of CNO-met-Hb from Hb-CN or Hb-N_3, while small amounts of NaCN or NaN_3 added to solutions of CNO-met-Hb promptly produced the respective CN- and N_3-compounds.

No attempt was made at a quantitative assessment of the respective affinities of these compounds.

No indication was found for a compound formation of carbamate with met-Hb. Incubated or old solutions of urea shifted the band in the red, because they contained cyanate, isomerized from urea[1, 3, 8, 9]. Freshly made solutions of urea did not influence the reaction of CNO′ with met-Hb. The fact that thiocyanate has less affinity for met-Hb than cyanate has been described by HECHT[4].

CHEMICAL TESTS

A number of chemical tests, known to affect certain haemoglobin derivatives, were carried out. In Table I it can be seen that CNO-met-Hb appeared more stable than met-Hb. Neither NH_4S, nor STOKES reagent converted it quantitatively into oxyhaemoglobin, as was the case with met-Hb. A certain, probably small, amount of CNO-met-Hb remained, even after addition of relatively large amounts of the reagents.

Hydrosulphite was similarly unable to convert all the CNO-met-Hb into deoxygenated Hb. Also after subsequent saturation with CO, and although an appreciable amount of CO-Hb was evidently formed, a small amount of CNO-met-Hb remained. While met-Hb is practically quantitatively converted into the respective compounds by the four mentioned procedures, their effect on CNO-met-Hb, though essentially similar, results in the setting up of an equilibrium between CNO-met-Hb and the respective reaction compound.

REVERSIBILITY OF THE REACTION OF CYANATE WITH METHAEMOGLOBIN

For the purpose of this question met-Hb may be regarded as a univalent base (HbOH) which dissociates in neutral or acid solution, thus:

$$HbOH \rightleftharpoons Hb^+ + OH^-$$

Obviously the hydroxyl group can be replaced by cyanate, as it is known to occur with various anions (CN, F, SH, etc.), giving rise to a saltlike compound, which in turn must be expected to set up other equilibria, one of which should be between the salt and the base, thus:

$$HbOH + CNO^- \rightleftharpoons HbCNO + OH^-$$
$$HbCNO \rightleftharpoons Hb^+ + CNO^-$$

The question of reversibility of the reaction of cyanate with met-Hb is of special interest with regard to other reactions of cyanate of possible physiological significance, *e.g.*, with proteins, amino acids, etc.[1, 10, 11]. In order to investigate this question, a sample of CNO-met-Hb was prepared by addition of a sufficient amount of NaCNO to

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TABLE I

THE EFFECT OF VARIOUS REAGENTS ON METHAEMOGLOBIN AND CYANATE-METHAEMOGLOBIN

The solutions of the pigments were suitably diluted in 0.08 *M*-phosphate buffer solution (pH 7.4), and the position of the absorption bands was determined with a HARTRIDGE reversion spectroscope, before and after the addition of the reagent.

Addition	Methaemoglobin	Cyanate-Methaemoglobin
1. —	6325 (strong)	6285 (very strong)
2. STOKES reagent, 0.1 ml	— 5762 5400 colour: red *oxyhaemoglobin*	6285 (definite) 5762 5400 colour: olive greenishred *mixture of oxyhaemoglobin and cyanate-methaemoglobin*
3. NH_4S, 5%, 0.05 ml	— 5762 5400 colour: red *oxyhaemoglobin*	6285 (faint) 5762 5400 colour: greenish red *mixture of oxyhaemoglobin and cyanate-methaemoglobin*
4. $Na_2S_2O_4$, 5%, 0.1–0.2 ml added in Thunberg tube, evacuated and filled with N_2	broad band 5400–5900 *deoxygenated ("reduced") haemoglobin*	6289 (definite) broad band 5400–5900. *mixture of deoxygenated haemoglobin and cyanate-methaemoglobin*
5. as (3), but saturated with CO	— 5690 5360 colour: cherry red *CO-haemoglobin*	6289 (definite) 5687 5360 colour: brownish red *mixture of CO-haemoglobin and cyanate-methaemoglobin*

a freshly prepared solution of met-Hb. The solution was left standing ½ h at room temperature. Thereafter it was dialysed through parchment or cellophane against distilled water at 4°, while the sack containing the solution was kept rotating by means of a motor. The distilled water was changed frequently. The band in the red was originally at 6285 Å, the position characteristic for CNO-met-Hb. On dialysis it moved towards 6325 Å, the position characteristic for met-Hb, without, however, reaching quite this point. *E.g.* after 24 h dialysis the readings were 6315–6320 Å.

When, in another experiment, erythrocytes containing met-Hb in the intact cells, were brought into contact with 0.9% NaCl solution, containing 0.05 *M*-NaCNO, the met-Hb inside the cells was rapidly transformed into CNO-met-Hb. When these cells were washed by repeated centrifugation and renewed suspension in fresh 0.9% NaCl solution, the band shifted again towards 6325 Å.

Both experiments, described above, show that the reaction of CNO with met-Hb is reversible.

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STABILITY

Solutions of met-Hb are well known to be rather unstable. On standing at room temperature, and more rapidly at 38°, the pigment agglomerates and precipitates, leaving a clear supernatant liquid. Solutions of CNO-met-Hb, however, are much more stable.

In one experiment a solution of met-Hb in 0.2 *M*-phosphate buffer solution (p_H 7.4) was prepared as usual; a solution of CNO-met-Hb, similarly buffered, was prepared from the same batch of met-Hb by addition of just the sufficient amount of NaCNO to convert practically all met-Hb present into CNO-met-Hb. Both solutions contained the pigment in similar concentration, and were of equal ionic strength. After 28 h incubation at 38° the solution of met-Hb was completely precipitated, showing a clear supernatant liquid. Of the CNO-met-Hb solution only a small part was precipitated, the supernatant was of the usual colour, and showed the absorption band in the red in the same position as before incubation.

DISCUSSION

The similarity of the effect of cyanate with that of caffeine, as described by J. KEILIN[12, 13] has already been discussed[1, 11].

The spectral changes brought about through the addition of cyanate to met-Hb are marked, the bands are shifted and reinforced but the main absorption bands are retained in very similar positions. Because of this great spectral similarity it seems appropriate to call the compound cyanate-methaemoglobin.

From recent studies it has become highly probable that small amounts of cyanate are being formed under physiological conditions[1, 3, 8, 9]. The data in the literature (BARCROFT *et al.*[14]), show, however, the position of the band in the red, of met-Hb circulating in the mammalian organism, at very much the same position as that of freshly made met-Hb, *i.e.*, not shifted towards the position of pure CNO-met-Hb. This does not exclude the possibility of cyanate being formed in the organism, because[1] cyanate may have, as some recent findings suggest[1, 10, 11] greater affinities for other substances (proteins, amino acids, cyanase, etc.) than for met-Hb. If only a small percentage of the circulating met-Hb would be transformed into CNO-met-Hb, it would not suffice to cause a significant shift of the band shown by the mixture of met-Hb and CNO-met-Hb. The bands of both compounds are so near each other, that even with the most sensitive small dispersion spectroscopes, only one single band is visible, when a mixture of both compounds is observed. In Fig. 1 it can be seen that, if only 10–20% are CNO-met-Hb, and the remainder met-Hb, no significant shift of the band was detectable; the small amount of CNO-met-Hb would be masked.

We are greatly indebted to the ELLA SACHS-PLOTZ-FOUNDATION, *Boston, U.S.A.*, for a grant to one of us (*F.S.*) as a substantial contribution towards the costs of purchase of a BECKMAN photoelectric spectrometer. We are also indebted to the *Medical Research Council* for a grant to one of us (*F.S.*) in aid of equipment of this laboratory, and to the *Mental Disease Research Board* of this University for financial assistance.

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SUMMARY

1. Details are given of the absorption spectrum of cyanate-methaemoglobin.
2. Cyanate was found to have a greater affinity for the iron of methaemoglobin than fluoride, but less than cyanide and azide.
3. The effects are described of STOKES reagent, ammonium sulphide, hydrosulphite and carbon monoxide on cyanate-methaemoglobin.
4. The red cell membrane is permeable for cyanate, since cyanate-methaemoglobin can be formed in intact cells containing methaemoglobin.
5. The reaction of cyanate with methaemoglobin was found reversible, by dialysis of the compound in aqueous solution against distilled water; or by repeated washings in 0.9% NaCl of cells containing the compound.
6. Cyanate-methaemoglobin was found considerably more stable than methaemoglobin. When the latter was precipitated on incubation at 38° (36 h), the former remained in solution.
7. The bearing is discussed of the results on the observed position of the absorption band in the red of circulating methaemoglobin.

RÉSUMÉ

1. Des détails sur le spectre d'absorption du composé cyanate-méthémoglobine sont indiqués.
2. Il a été trouvé que le cyanate a pour le fer de la méthémoglobine une affinité plus grande que le fluorure, mais plus petite que le cyanure et l'azides.
3. On donne une description des effets du réactif de STOKES, du sulfure d'ammonium, des hydrosulfites et de l'oxyde de carbone sur le composé cyanate-méthémoglobine.
4. La membrane des cellules d'érythrocytes est perméable à l'ion cyanique, puisque le composé cyanate-méthémoglobine peut être formé dans des cellules intactes contenant de la méthémoglobine.
5. La réaction de l'ion cyanate avec la méthémoglobine est réversible; on peut le démontrer soit par dialyse du composé en solution aqueuse contre de l'eau distillée, soit par des lavages répétés des cellules contenant ce composé avec une solution de NaCl à 0.9%.
6. Le composé cyanate-méthémoglobine s'est révélé beaucoup plus stable que la méthémoglobine. Alors que celle-ci est précipitée par incubation à 38° pendant 36 heures, celui-là reste en solution.
7. L'incidence de ces résultats sur la position observée de la bande d'absorption dans le rouge de la méthémoglobine circulant dans l'organisme animal a été discutée.

ZUSAMMENFASSUNG

1. Einzelheiten werden mitgeteilt über das Absorptionsspektrum von Cyanat-methämoglobin.
2. Es wurde gefunden dass Cyanat eine stärkere Affinität zum Eisen des Methämoglobins besitzt als Fluorid, jedoch eine schwächere als Cyanid und Azid.
3. Die Einwirkungen von STOKES' Reagens, Ammoniumsulfid, Hydrosulphit und Kohlenmonoxyd auf Cyanat-methämoglobin werden beschrieben.
4. Die Membran der roten Blutkörperchen ist für Cyanat durchblässig, da Cyanat-methämoglobin in unbeschädigten, Methämoglobin enthaltenden Zellen gebildet werden kann.
5. Die Reaktion von Cyanat mit Methämoglobin ist umkehrbar; dies wurde festgestellt durch Dialyse der Verbindung in wässriger Lösung gegen destilliertes Wasser, oder durch wiederholtes Waschen der die Verbindung enthaltenden Zellen mit 0.9%iger NaCl-Lösung.
6. Es wurde festgestellt, dass Cyanat-methämoglobin bedeutend stabiler ist als Methämoglobin. Während Letzteres bei 36 stündiger Inkubation bei 38° niedergeschlagen wurde, blieb Ersteres in Lösung.
7. Die Auswirkung der Ergebnisse auf die beobachtete Lage der Absorptionsbande im Roten von im Tierkörper zirkulierendem Methämoglobin wird besprochen.

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