values for sodium and potassium are known. The effect of phosphate is also small.

The introduction of 16 % (v/v) isopropanol in the diluted samples increased the single-cell responses by 70 % (see Alkemade, Smith & Verschure, 1951), but conferred no advantage in the internal-standard method. Fig. 8 shows the effect of acetylene flow-rate on calcium determinations made with the single cell and the internal standard methods.

DISCUSSION

The photometric determinations described give results which agree well with those obtained titrimetrically, and which show an average difference from theoretical of less than $\pm 2\%$ (Table 1). They are obtained rapidly and without elaborate preparation of the sample; measurements of serum calcium by the internal-standard method are much to be preferred to those made with a single cell.

Although it is clear that use of an internal standard enhances stability and reproducibility (Spencer, 1950; Bernstein, 1952), it is essential to ensure that the conditions in which it is used are optimum. Failing this, use of the method may introduce errors greater than those occurring in single-cell determinations (Fig. 8).

Of the bands in the calcium flame-spectrum, that at 6200 Å is detected most sensitively by the photoconductive cells, whose characteristic curve (Schwarz, 1950) shows a peak at 7200 Å. With given illumination these cells pass current proportional to the potential applied, which may be up to 100 v. Thus the sensitivities of a pair of cells may be matched easily by choice of potentials. The ultimate sensitivity (Schwarz, 1951) is of the same order as that of a photomultiplier tube.

SUMMARY

An internal standard flame photometer for estimation of calcium, sodium and potassium in serum,

with dilution as the only pretreatment of the sample, is described, and some results are reported.

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Reactions of Free Haematin and Haemoproteins with Nitric Oxide and certain other Substances

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The reversible combination of nitric oxide with haemoglobin was discovered in 1865 by Hermann, who observed the changes not only in the colour but also in the absorption spectrum of a solution of haemoglobin treated with nitric oxide in the complete absence of oxygen. By careful gasometric experiments he showed that haemoglobin

combines with the same amount of nitric oxide as of carbon monoxide and that the affinity of haemoglobin for nitric oxide was greater than for carbon monoxide, and therefore much greater than for oxygen.

It was claimed by Linossier (1887) that free haematin also combines with nitric oxide. This reaction, which, as he mentions himself, was discovered by his students, was not clearly described

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by him and its occurrence was later dismissed by Gamgee (1898) in his classical review of haemoglobin and its derivatives. However, according to Gamgee, nitric oxide appeared to form with 'haemochromogen' a compound which resembled oxyhaemoglobin spectroscopically, and he concluded that 'this NO-haemochromogen awaits careful examination'. It must be mentioned here that at that time Gamgee had no clear view as to the relationship between reduced haematin (haem) and 'haemochromogen', terms which were then often used for the same substance, or of the valency of the iron in the 'haemochromogen'.

The absolute spectrophotometric curve of NO-haemoglobin was given by Haurowitz (1924, 1926) and by Drabkin & Austin (1935), who confirmed that the affinity of haemoglobin is much greater for nitric oxide than for oxygen. Nitric oxide was found to form a less stable compound with met-haemoglobin, the absorption spectrum of which shows marked differences from that of NO-haemoglobin (Keilin & Hartree, 1937).

The magnetic properties of NO-haemoglobin determined by Coryell, Pauling & Dodson (1939) show that the iron atoms in this compound are involved in octahedral-bond formation, having one unpaired electron per haem owing to the odd number of electrons in the NO-group.

The object of the present investigation is to show that free haematin (ferric-porphyrin) and the corresponding haems (ferro-porphyrin) do combine with nitric oxide, under certain well-defined conditions, and to compare NO-haematin or haem with the NO-haemoprotein compounds.

The study of these compounds is followed by a comparative examination of the reactivities of free Fe-porphyrin and of the Fe-porphyrin prosthetic group of the natural haemoproteins towards a number of different substances which include both the substrate and the well-known reversible inhibitors of the biological activities of these haemoproteins.

EXPERIMENTAL

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

Urohaemin. This was prepared by the method of Fischer & Orth (1934) from the octamethyl ester of uroporphyrin I, the free porphyrin having been isolated from the urine of a case of congenital porphyria. Urohaemin was used for these studies in addition to protohaemin, since it has been shown that, owing to the greater solubility conferred upon urohaemin by its eight carboxyl groups, it reacts with some substances far more readily than protohaemin (J. Keilin, 1949b, 1950).

Acetic acid. Twice-redistilled A.R. acetic acid was used.

Nitric oxide. The gas was prepared by heating a mixture of NaNO_2 and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in dilute H_2SO_4 , and was freed from all traces of NO_2 by passing it over pumice stone soaked in H_2SO_4 . It was collected over mercury in

a gasometer (Hartree & Harpley, 1949) all connexions having been previously filled with O_2 -free nitrogen.

Gassing of solutions. The solution under investigation was contained in a hollow-stoppered Thunberg tube. For spectrophotometric work a special Thunberg tube was used, the lower end of which consisted of an optical glass cell of 0.5 cm. light path. The tube was evacuated, flushed out twice with O_2 -free nitrogen and once with NO before being finally filled with NO. The tube was well shaken throughout the process in order to equilibrate the solution with the gas phase at each stage.

Spectroscopic methods. A microspectroscope was used for the direct observations of the reactions. The absorption spectra in the visible and ultraviolet regions were determined with the Beckman photoelectric spectrophotometer using the Thunberg tube described above. Since only one such tube was available, a 0.5 cm. optical glass cell containing the same reagents as the Thunberg tube (except for the haematin and the gas under investigation) was used as the blank. As the heights of the bands of the NO-haematin compounds tended to fall a little with time, fresh solutions were used for the visible and Soret regions of the spectrum respectively and the first determinations of the extinctions were made in the regions of the peaks of the absorption bands without delay since these extinctions are the most liable to change.

The molecular extinction coefficient ϵ is defined as follows: $\epsilon = E/cl$, where c = molar concentration of haemin, l = optical depth in cm., and E (extinction) = $\log I_0/I$, where I_0 and I are the intensities of the incident and transmitted light respectively.

RESULTS

Reactions of uro- and proto-haematin with nitric oxide

When a $6.16 \times 10^{-4}\text{M}$ solution of urohaematin in 0.02N or 0.1N-NaOH is saturated with nitric oxide in a Thunberg tube in the absence of oxygen, the colour of the urohaematin immediately changes from reddish brown to a bright red, and direct spectroscopic observation shows that the single diffuse absorption band of alkaline urohaematin at 595 m μ . is replaced by two sharp absorption bands of equal intensity lying at about 563 and 528 m μ . These absorption bands are due to the formation of the alkaline NO-urohaematin compound. The absorption curves (Figs. 1 and 2) show, however, that under these conditions the urohaematin does not all react, the residual free urohaematin giving rise to a low band at about 600 m μ . and to the shoulder at 396 m μ . on the short-wave side of the Soret band which lies at 416 m μ . A similar type of absorption spectrum was noted in acid solution (0.006N acetic acid) but the optimum conditions for the formation of NO-urohaematin appeared to be in glacial acetic acid (urohaematin is precipitated by mineral acids). The spectrophotometric curves (Fig. 3) show that the absorption spectrum of acid NO-urohaematin consists of a sharp α -band at 558 m μ . and a lower β -band at 525 m μ ., the spectrum in the visible region resembling that of a

haemochromogen. The Soret band (Fig. 4) lies at $410\text{ m}\mu$. but it is complicated by the presence of five small additional absorption bands lying at 389 , 373.5 , 359.5 , 347.5 and $337\text{ m}\mu$. respectively. The origin of these bands will be discussed later. Urohaematin did not appear to react with NO in neutral solution.

Protohaematin was found to react similarly to urohaematin with NO in acid solution and the same five additional bands described above were found in the ultraviolet region (Figs. 5, 6). Direct observa-

tion with the microspectroscope showed that protohaematin in 0.007 N-NaOH gave with NO a diffuse absorption band which showed two ill-defined maxima at about 570 and $535\text{ m}\mu$. but spectrophotometric curves of the brownish pink solution showed that there was only a single very broad plateau-like band extending from 575 to $535\text{ m}\mu$., the relatively steep sides of this plateau giving the impression of two maxima. Under more alkaline conditions (about 0.05 N-NaOH) the rose-coloured solution became opalescent and two diffuse absorption

Fig. 1. Absorption spectra in the visible region of urohaematin, NO-urohaematin and NO-urohaem in 0.1 N-NaOH . For details, see legend to Table 1.

Fig. 2. Absorption spectra in the near ultraviolet of urohaematin, NO-urohaematin and NO-urohaem in 0.1 N-NaOH . For details, see legend to Table 1.

Fig. 3. Absorption spectra in the visible region of urohaematin and NO-urohaematin in glacial acetic acid. For details, see legend to Table 1.

Fig. 4. Absorption spectra in the near ultraviolet of urohaematin and NO-urohaematin in glacial acetic acid. The extinction of glacial acetic acid saturated with NO is shown below (ordinate scale on right). For details, see legend to Table 1.

bands appeared at about 570 and 535 $m\mu$. of which the α -band was the stronger.

In 0.007*N*-NaOH and in the presence of a few mg. of caffeine or 20–50% (v/v) ethanol as solubilizing agents, protohaematin gave with NO a clear rose-red solution with diffuse absorption bands in the visible region ($\epsilon \times 10^{-4}$ at 570 $m\mu$. = 0.72; $\epsilon \times 10^{-4}$ at 538 $m\mu$. = 0.76) and a broad low Soret band centred at 395 $m\mu$. ($\epsilon \times 10^{-4}$ = 4.6). This absorption spectrum bears some resemblance to that of protohaem (Fe^{3+}) but the ferric nature of the haematin in this NO compound may be shown by replacing the NO in the Thunberg tube with carbon

monoxide: no CO compound is formed until the haematin iron is reduced by the addition of sodium dithionite ($Na_2S_2O_4$).

Both uro- and proto-haematin reacted with nitric oxide generated in the solution by the action of acetic acid on a few mg. of sodium nitrite. The absorption bands of the NO-haematin compounds were, however, slightly displaced and their extinction coefficients were a little lower than those of the absorption bands produced by treating the haematin with NO from the gasometer (Table 1). However, the use of acidified sodium nitrite for qualitative work enables experiments to be carried out in tubes and cells exposed to air.

Fig. 5. Absorption spectra in the visible region of protohaematin and NO-protohaematin in glacial acetic acid. For details, see legend to Table 1.

Fig. 6. Absorption spectra in the near ultraviolet of protohaematin and NO-protohaematin in glacial acetic acid. The extinction of glacial acetic acid saturated with NO is given below (ordinate scale on right).

Table 1. Positions and extinction coefficients of the α -, β - and γ -bands of compounds of urohaematin and protohaematin with and without NO

Urohaematin $7.7 \times 10^{-5}M$ for visible spectrum: $1.54 \times 10^{-5}M$ for near u.v.: 0.5 cm. light path. Protohaematin 1.05×10^{-4} and $2.1 \times 10^{-5}M$ resp., except for the spectrum of protohaem in 0.1*N*-NaOH where the concns. were 1.31×10^{-4} and $1.31 \times 10^{-5}M$ for the visible and u.v. spectra: light path 1.0 cm. Where applicable: Gas phase, 1 atm. NO; $NaNO_2$, 5 to 10 mg./ml.; caffeine, about 5 mg./ml.; $Na_2S_2O_4$, least amount giving reduction (about 1 mg./ml.). Molecular extinctions (ϵ) calculated on the basis of the total haematin present as defined in the text.

Compound	α -band		β -band		γ -band	
	Wavelength ($m\mu$.)	$\epsilon \times 10^{-4}$	Wavelength ($m\mu$.)	$\epsilon \times 10^{-4}$	Wavelength ($m\mu$.)	$\epsilon \times 10^{-4}$
Urohaematin in AcOH	620	0.39	498	0.695	395	9.5
Urohaematin in AcOH + NO	558	1.62	525	1.26	410	15.8
Urohaematin in AcOH + $NaNO_2$	560	1.58	528	1.17	410.5	12.75
Urohaematin in 0.1 <i>N</i> -NaOH	595	0.75	483	0.82	396	10.3
Urohaematin in 0.1 <i>N</i> -NaOH + NO	562.5	1.07	528	1.08	416	11.6
Urohaematin in 0.1 <i>N</i> -NaOH + NO + $Na_2S_2O_4$	568*	0.83	543	1.48	388	11.55
Dihydroxyl urohaem†	578	0.76	546	1.4	435	11.0
Protohaematin in AcOH	633	0.38	505*	0.85	383	8.5
Protohaematin in AcOH + NO	563	1.68	528	1.215	414	10.0
Protohaematin in AcOH + $NaNO_2$	561	1.29	528	1.03	415.5	10.45
Protohaematin in 0.1 <i>N</i> -NaOH	610	0.46	—	—	384	5.1
Protohaematin in 0.007 <i>N</i> -NaOH + caffeine + NO	570	0.72	538	0.76	395	4.6
Protohaem in 0.1 <i>N</i> -NaOH	565	0.61	—	—	390	3.96

* Shoulder on slope of main band.

† J. Keilin (1949*b*).

The reduced form of NO-urohaematin

As the iron of haematin is most easily reduced by sodium dithionite in alkaline solution, all experiments on the effect of NO on haem (Fe^{2+}) were carried out in the presence of sodium hydroxide.

Urohaematin in 0.1N-NaOH was treated with NO in a Thunberg tube containing a few mg. $\text{Na}_2\text{S}_2\text{O}_4$ in the hollow stopper. As soon as the red NO-urohaematin was formed, it was reduced by inverting the tube several times and thoroughly mixing the contents with the $\text{Na}_2\text{S}_2\text{O}_4$. The NO-urohaem thus obtained has a very broad but strong absorption band, the centre of which lies at 543 m μ . (Fig. 1), while the Soret band lies at 388 m μ . (Fig. 2). It should be noted that in the absence of NO, reduction of urohaematin in 0.1N-NaOH would give rise, not to urohaem with its single diffuse absorption band in the visible region, but to dihydroxyl-urohaem. This compound has two very well-defined bands of which the β -band at 546 m μ . is stronger than the α -band at 578 m μ ., while the Soret band lies at 435 m μ . (J. Keilin, 1949b).

Reversibility of NO-compound formation with haematin and haem

The reversibility of the reaction between NO and urohaematin can be demonstrated by preparing NO-urohaematin in glacial acetic acid in a Thunberg tube as described earlier in this paper, and then evacuating the tube with constant shaking. The bright red colour of NO-urohaematin gradually turns to brown and after 30 min. evacuation the solution shows only the absorption bands of acid urohaematin. The dissociation of NO-urohaematin was followed directly by placing two portions of a solution of urohaematin in glacial acetic acid in wide, flat-bottomed, open glass tubes. A few mg. of NaNO_2 were added to each tube and as soon as visible gas production in the solutions had ceased, their absorption spectra were examined with the microspectroscope from above through the full depth of fluid. On the addition of a large excess (10–20 vol.) of glacial acetic acid to one tube the colour of the solution changed from red to brown while the absorption bands of NO-urohaematin became progressively weaker as the spectrum of free acid urohaematin reappeared.

In alkaline solution reversibility of the reaction with NO is best shown by treating a solution of alkaline urohaematin in a Thunberg tube with a gas mixture consisting of 20% (v/v) NO and 80% (v/v) N_2 . A few mg. of $\text{Na}_2\text{S}_2\text{O}_4$ are added from the hollow stopper as described above. The tube is well shaken and the absorption bands of NO-urohaem are seen, but when the solution is allowed to stand for a few minutes these bands are replaced by the bands of dihydroxyl-urohaem. The NO-urohaem

spectrum may be restored by shaking the tube vigorously, but on standing the dihydroxyl-haem spectrum soon returns. The most likely explanation of these observations is that part of the small amount of NO that dissolves in the solution from this gas mixture combines with the urohaem. The rest of the NO in solution may react with the alkali present and perhaps also with the dithionite to give nitrous oxide and sodium nitrite. This alters the equilibrium and causes the NO-urohaem to dissociate, leaving the urohaem free to combine with NaOH to give dihydroxyl-haem. Vigorous shaking causes more NO to pass from the gas to the liquid phase and the cycle is repeated.

If, however, pure NO is used instead of the gas mixture described, there is sufficient NO dissolved in the solution to prevent any dissociation of the NO-urohaem compound.

The nature of the additional absorption bands in the ultraviolet region

The additional absorption bands found between 390 and 335 m μ . in solutions of acid haematins treated with nitric oxide were found to vary in intensity with each preparation (Figs. 4, 6). It was observed that whereas the height of the Soret band tended to fall with time, the additional bands gradually increased in intensity. Moreover, their positions were constant and independent of the position of the Soret band which of course is determined by the nature of the haematin, the absorption bands of protohaematin and its derivatives lying at longer wavelengths than the corresponding bands of urohaematin compounds. (It must be remembered that owing to the apparatus available neither NO nor NaNO_2 was present in the open control cell.) Control experiments with acetic acid alone, or nitric oxide alone showed no absorption bands in this region but glacial acetic acid or $\text{N-H}_2\text{SO}_4$ saturated with nitric oxide gave a five-banded absorption spectrum which gradually increased in intensity (Table 2). The absorption spectrum of NO-saturated acetic acid which had been allowed to stand at room temperature for 11 days is shown in Figs. 4 and 6, where the extinction E is plotted against wavelength, and it can be seen that in the acid NO-haematin solutions similar absorption bands are superimposed on the slope of the Soret band. In the case of acid NO-haematin where the nitric oxide was formed by the decomposition of sodium nitrite in the solution, these additional absorption bands are very strong, the band at 389 m μ . being of the same height as the Soret band. It was thought that these absorption bands might be due to the reaction of NO with traces of oxygen dissolved in the solution to give NO_2 , but the absorption spectrum of NO_2 (Brewster, 1834) consists of hundreds of very fine lines distributed

Table 2. *Positions and relative heights of absorption maxima and minima given by nitric oxide in acetic acid and N-H₂SO₄*

Acid	Max. I		Min. I		Max. II		Min. II		Max. III	
	(m μ .)	E_I^*	(m μ .)	$\frac{E_{min.}}{E_I}$	(m μ .)	E_{II}	(m μ .)	$\frac{E_{min.}}{E_{II}}$	(m μ .)	E_{III}
AcOH	389	1.0	381	0.431	373.5	1.58	365	0.39	359.5	1.43
N-H ₂ SO ₄	386	1.0	381	0.87	372	1.51	366	0.74	358.5	1.46

Acid	Min. III		Max. IV		Min. IV		Max. V	
	(m μ .)	$\frac{E_{min.}}{E_{III}}$	(m μ .)	E_{IV}	(m μ .)	$\frac{E_{min.}}{E_{IV}}$	(m μ .)	E_V
AcOH	352	4.25	347.5	0.97	340	0.477	337	0.535
N-H ₂ SO ₄	352.5	6.73	340	1.05	—	—	—	—

* An arbitrary value of 1.0 is assigned to E for the first maximum and the extinctions of the other bands are expressed relatively to it.

throughout the whole spectrum, so the five absorption bands encountered here are most likely due to some other nitrogenous derivative formed by nitric oxide in acid solution.

Effect of azide, fluoride and sulphide on proto- and uro-haematin

Sodium azide, sodium fluoride and sodium sulphide which, like nitric oxide, so easily combine with methaemoglobin and other haemoproteins were each added to solutions of protohaematin and urohaematin under a great variety of conditions and the absorption spectra of the solutions were observed with the microspectroscope. No changes in the absorption spectra of the haematin attributable to compound formation between the haematin and these substances were seen when examined in phosphate buffers covering a wide range of pH, in 0.1 N-NaOH, 0.1 N-KH₂PO₄ or in acetic acid. This does not, however, necessarily rule out the possibility that such compounds might be formed under some other conditions which have yet to be found.

Reactions of myoglobin with hydrazine and pyridine

It was shown by Schumm (1925) that whereas haemoglobin treated with hydrazine rapidly forms a typical haemochromogen with its α -band at 558 m μ ., myoglobin treated in the same way has a many-banded absorption spectrum consisting of two sharp bands at 566 and 554 m μ . and two diffuse bands at 536 and 528 m μ . A band at 582 m μ . may precede the development of this spectrum but is usually seen for only a short time. These findings were confirmed by Bechtold (1935) and a similar absorption spectrum was obtained by Kiese & Kaeske (1942) on treating myoglobin with hydrazine and pyridine and by Gonella & Vannotti (1943), who treated myoglobin with dilute aqueous pyridine. According to these workers the absorption spectrum belongs to two compounds; the

one having its α -band at 565 m μ . differing from myoglobin in that the Fe, while remaining attached to globin, is also co-ordinated with a molecule of pyridine. The second compound having its α -band at 554 m μ . is an ordinary pyridine haemochromogen in which the haem iron is linked with two molecules of pyridine. This haemochromogen they believe to be formed from free protohaem adsorbed on to the myoglobin.

In the course of the present study the observations made by the above workers on the reactions of myoglobin with both pyridine and hydrazine were confirmed. Thus when a solution of myoglobin is treated with pyridine, two α -bands are seen, of which the band at 565 m μ . is always much stronger than the one at 554 m μ . The ultraviolet region of the spectrum shows a broad asymmetric Soret band with its maximum at 423 m μ . The shoulder on the longer wavelength side of this band is probably due to a band centred at about 434 m μ . It seems reasonable to assume that the band at 423 m μ . is the Soret band of the compound possessing the stronger absorption spectrum (α -band at 565 m μ ., β -band at 536 m μ .) while the band at 434 m μ . is the Soret band of the compound possessing the weaker spectrum (α -band at 554 m μ ., β -band at 528 m μ .). A band at 580 m μ . which is in the position of the α -band of oxymyoglobin is often seen together with the other bands and can be made more intense by shaking the solution vigorously so as to aerate it.

It is suggested that in the compound responsible for the absorption spectrum with an α -band at 565 m μ . the haem remains attached to the native globin through both the porphyrin and the Fe. The 5th co-ordination bond of the iron atom unites it with a haem-binding group of the globin, while the 6th bond links it to a molecule of pyridine instead of water as in the case of myoglobin. This seems to be a probable explanation of the absorption spectrum and the compound, which can be con-

sidered as a 'hybrid haemochromogen', is very similar to cyan-haemoglobin or cyan-myoglobin obtained by reducing cyan-methaemoglobin or cyan-metmyoglobin respectively. On the other hand, the compound with the α -band at 554 or 555 m μ . is probably a pyridine haemochromogen (two molecules of pyridine co-ordinated with the haem Fe, one on either side of the flat haem molecule) in which the haem remains attached through its porphyrin ring to the native globin. This explanation is supported by the fact that the absorption spectrum of this compound is given by twice-recrystallized myoglobin, which is devoid of free haem, and the absorption bands are nearer the blue end of the spectrum than those of an ordinary pyridine haemochromogen (α -band at 558 m μ .). It is well known that the attachment of a protein molecule to a free porphyrin or to the porphyrin ring of a haem molecule brings about a greater state of dispersion of the pigment with a resultant shift of the visible absorption bands towards the blue end of the spectrum (Hill & Holden, 1926; Haurowitz & Waelsch, 1929).

DISCUSSION

It is well known that nitric oxide combines reversibly with haemoglobin and methaemoglobin. As to its reactions with the iron of the free prosthetic groups of these haemoproteins, the information so far available was very incomplete and contradictory. The experiments described in this paper have established that nitric oxide reacts reversibly with both free haem and haematin, forming compounds the absorption spectra of which are recorded here for the first time. Thus nitric oxide has the property of combining with the bivalent and trivalent iron of haem and haematin respectively both when they are free and when, as prosthetic groups, they form part of haemoprotein molecules. Of all the other nitrogenous substances known to react with haemoglobin and its derivatives, this remarkable property of nitric oxide is shared only by cyanide. On the other hand, cyanide in its reactions with free haem differs from all other nitrogenous substances in that it can form two distinct compounds containing one and two CN' groups per haem iron respectively (Anson & Mirsky, 1928; Hill, 1929; J. Keilin, 1949*a*).

In the case of the haemoproteins, cyanide combines most readily with the ferric iron of methaemoglobin giving a very stable cyan-methaemoglobin complex. When the complex is reduced with sodium dithionite, its characteristic absorption band at 540 m μ . is replaced by two bands at about 562 and 535 m μ . This haemochromogen-like absorption spectrum belongs to cyan-haemoglobin, a hybrid cyan-native-globin haemochromogen. The

compound is unstable, and on standing soon gives rise to ordinary haemoglobin (Balthazar & Philippe, 1926; Anson & Mirsky, quoted by Stitt & Coryell, 1939). Cyan-myoglobin, on the other hand, is much more stable (Kiese & Kaeske, 1942) and can be obtained not only by reducing cyan-metmyoglobin but also directly by treating myoglobin with cyanide. The magnetic properties of cyan-haemoglobin were determined by Stitt & Coryell (1939), who found it to be diamagnetic, having the iron linked to the surrounding atoms by covalent bonds.

Although isocyanide (carbylamine), like cyanide and nitric oxide, combines reversibly with the bivalent iron of haem and haemoglobin (Warburg, Negelein & Christian, 1929), it differs from cyanide and nitric oxide in that it does not combine with the trivalent iron of haematin and methaemoglobin.

The reactions of cyanide and nitric oxide with haemoglobin and its derivatives are of special interest considering that other substances are so far known to react either with the free prosthetic group or only with the haemoprotein and when they do react with both, it is only when their iron is in either the bivalent or trivalent state. Substances which are capable of reacting with haems, haematins and haemoproteins can therefore be classified as shown in Table 3 into three groups, *A*, *B* and *C*. In this table, in which only the more important reactions are considered, the term iron-porphyrin includes protohaematin, which is the prosthetic group of nearly all natural haemoproteins, and urohaematin which is more soluble than protohaematin and has no tendency to polymerize or aggregate. Of the haemoproteins only haemoglobin and myoglobin are considered here, although other haemoproteins such as catalase and peroxidase have many properties in common with methaemoglobin (Keilin & Hartree, 1951).

The reactivities of free Fe-porphyrins and of haemoproteins with the substances recorded in this table will be considered here only in terms of their co-ordination with the iron atom. In this respect it is important to recall that haematin is a flat molecule, the iron atom fitting tightly in the centre of the porphyrin ring. Of the six co-ordination bonds of the iron atom, four lie in the plane of the molecule and join the iron to the nitrogen atoms of the four pyrrol nuclei; the other two bonds (5th and 6th), lying one on each side of the flat haematin molecule, link the iron to two other groups which are: one molecule of water and one hydroxyl group in alkaline haematin (Fe³⁺), two molecules of water in haem (Fe²⁺) (Davies, 1940) and two molecules of a nitrogenous base in the parahaematins (Fe³⁺) and in the haemochromogens (Fe²⁺).

In haemoglobin and methaemoglobin, the 5th co-ordination bond is occupied by a nitrogenous group of the protein while the 6th joins the iron

atom to a molecule of water (Haurowitz, 1951). The latter, in haemoglobin, can be replaced by a molecule of oxygen, carbon monoxide, carbylamine, etc., while in methaemoglobin it may be replaced by a hydroxyl, fluoride, cyanide, azide or sulphide group. The electronic configuration of the iron atom precludes it from co-ordinating with more than six groups but this does not prevent the porphyrin side-chains in free haematin, or some groups of the protein in the haemoprotein molecule, from combining with different substances. In the case of a haemoprotein, although the formation of such a compound might affect to some extent the affinity of the haematin iron atom towards certain substances, it has no effect on the absorption spectrum of the haemoprotein, which is determined by the valency of the iron, the nature of the bonds uniting it with surrounding atoms and the nature of the substances coordinating with it.

The study of the reactions shown in Table 3 suggests a number of interesting problems but only a few of them can be discussed here. One may ask why substances of group A, which combine so readily with free haem or haematin, do not appear to combine with haemoproteins? The failure of the haemoprotein iron to react with these substances may be due partly to steric hindrance, as might be expected in the case of the larger molecules such as denatured proteins, polypeptides or even nicotine and partly to the tendency of these substances to denature the haemoproteins when used in concentrations high enough to react with the haem iron. In fact it has been shown that myoglobin, which is

less easily denatured than haemoglobin, reacts reversibly with hydrazine and with pyridine. However, the concentrations of the latter substances required for this reaction are much higher than the concentrations at which they combine with free haem and haematin to give the corresponding haemochromogen and para-haematin compounds respectively. On the other hand, no satisfactory explanation can be offered to account for the fact that azide and sulphide and fluoride, which combine so readily with the tervalent iron of the haemoproteins as well as with free iron, fail to react with the iron of free haematin. It is possible that these substances may react with free haematins under conditions that have not yet been found. That such a possibility cannot be eliminated at this stage is indicated by the very special conditions which were required for the reactions of free haematins with certain amino acids and peroxides (J. Keilin, 1950, 1952) and, as was shown in the present paper, with nitric oxide. It is also difficult to explain why, of all the substances so far examined, only nitric oxide and the cyanide ion are capable of co-ordinating with the bivalent and tervalent iron of haem and haematin respectively both when they are free and when, as prosthetic groups, they form part of the haemoproteins. A proper understanding of the reactions discussed above may help in elucidating the mechanism of the interactions between native proteins and their haematin prosthetic groups whereby the molecule may acquire the properties of an oxygen carrier or of a very active oxidizing catalyst.

Table 3. *Reversible reactions of free Fe-porphyrins and haemoproteins with different substances*

Pigment	Substance co-ordinating with Fe	Valency of Fe
I <i>Free Fe-porphyrin</i> Haem (Fe ²⁺) Haematin (Fe ³⁺)	→ A* { Ammonia, nicotine, piperidine, pyridine†, hydrazine hydrate†, amines, amino acids, polypeptides, denatured proteins and many other nitrogenous substances }	2⇌3
	→ B { Carbon monoxide Nitrosobenzene IsoCyanide Cyanide Nitric oxide Imidazole Hydrogen peroxide Ethyl hydroperoxide }	2 2 2 2⇌3 2⇌3 2⇌3 (I); 3 (II) 3 3
II <i>Haemoproteins</i> Haemoglobin } Fe ²⁺ Myoglobin } Methaemoglobin } Fe ³⁺ Metmyoglobin }	→ C { Oxygen Azide Fluoride Sulphide }	2 3 3 3

* Compounds formed: haemochromogen (Fe²⁺)⇌para-haematin (Fe³⁺).

† Also react with myoglobin; see text, p. 576.

SUMMARY

1. It is shown that free haematins will react with nitric oxide under certain conditions giving compounds with characteristic absorption spectra.

2. The reactions of nitric oxide with both protohaematin and urohaematin were examined. In acid solution each of these haematins changes colour from reddish brown to a bright red in the presence of nitric oxide, and two strong absorption bands appear in the green region of the spectrum while the Soret band of the haematin is shifted to a longer wavelength.

3. Urohaematin in alkaline solution reacts similarly with nitric oxide and can be reduced to give a NO-urohaem compound with a single broad absorption band in the visible region. The results obtained with alkaline protohaematin are less clear-cut.

4. The absorption spectra of the NO-compounds of proto- and uro-haematin under various conditions have been determined.

5. No spectroscopic evidence was obtained for any reaction between fluoride, azide or sulphide with free haematins, although these substances all form well-defined compounds with methaemoglobin and other haemoproteins.

6. The reactions of different substances with free iron-porphyrins were compared with those given with haemoproteins. Of all the substances examined only nitric oxide and the cyanide ion are capable of co-ordinating with the bivalent and trivalent iron of haem and haematin respectively, both when they are free and when they form the prosthetic groups of haemoproteins.

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Glutamine Synthesis by *Micrococcus pyogenes* var. *aureus*

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The synthesis of glutamine has been studied by Elliott (1948, 1951) and Speck (1949) in cell-free extracts of sheep brain and pigeon liver. Synthesis of glutamine occurred when the enzyme preparation was incubated with ammonia, magnesium ions, L-glutamic acid and adenosine triphosphate (ATP). Replacement of ammonia by hydroxylamine led to the synthesis of a hydroxamic acid, which by analogy with glutamine was presumably γ -glutamyl-

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hydroxamic acid. Since hydroxamic acids can be readily detected and estimated by the formation of a coloured complex in the presence of ferric ions at an acid pH (Lipmann & Tuttle, 1945), synthesis of glutamylhydroxamic acid is a convenient test for detecting the ability of an enzyme preparation to synthesize glutamine. Concurrent with Elliott's studies of the sheep-brain system, Elliott & Gale (1948) showed that cell-free extracts of *Micrococcus pyogenes* var. *aureus* synthesized glutamylhydroxamic acid. The work now described presents