3. *Range of method*

The range of the method for the blood sample which includes the internal control is 300 mg./100 ml., but for other blood samples done at the same time it is 500 mg./100 ml.

RESULTS

Results obtained with the present method and with the Fujita & Iwatake [1931] modification of the Hagedorn-Jensen procedure on the same blood specimens are in good agreement. Those for the microdiffusion procedure are a little lower on the average, as might be expected, and the difference is more marked in the fasting subject. Table 2 gives a summary of the results. All determinations were made on oxalated blood, immediately after the specimen had been taken.

Table 2. *Comparison of blood sugar methods*

	Hagedorn-Jensen method (modification of Fujita & Iwatake) mg./100 ml.	Microdiffusion method mg./100 ml.
Non-fasting subjects		
1	103	108
2	137	128
3	110	112
4	154	157
5	99	95
6	107	94
Mean	118	118
Fasting subjects		
7	106	111
8	113	101
9	89	75
10	96	75
Mean	101	91
Weighted mean for the two groups	111	106

DISCUSSION

The present microdiffusion method involves less manipulation and fewer solutions than procedures such as the Hagedorn-Jensen, and is very suitable for a large number of determinations. Application of the method to a more complete investigation of the true glucose concentration of the blood, and to normal and pathological urines, etc., is still in an experimental stage, but the method for blood glucose appears sufficiently well developed for clinical use.

It should be noted that anti-glycolytic measures must not be used in collecting the blood, and that the method is independent of glycolysis up to 5 hr. at room temperature, or for one whole working day if the specimens are stored in the refrigerator.

SUMMARY

1. A microdiffusion method is described for the determination of blood glucose using 0·1 ml. samples. A smaller microdiffusion unit than the standard is used (Conway unit, no. 2).

2. The results obtained are very similar to those obtained with the Hagedorn-Jensen method modified by substitution of the Fujita & Iwatake [1931] procedure of deproteinization with cadmium instead of zinc, and which is claimed to give a measure of the true blood glucose.

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Effect of Caffeine and other Iminazole Compounds on Haematins and their Derivatives

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It is well known that haem combines reversibly with a great variety of nitrogenous substances [Anson & Mirsky, 1925], giving the corresponding haemochromogens. This reaction is recognized by the replacement of the reddish brown colour and diffuse absorption bands of haem by the red colour and very sharp bands of haemochromogen.

By the analysis of crystalline haemochromogens [Zeynek, 1910] and by spectroscopic titration [Hill, 1926] it was shown that haemochromogens are compounds of haem with two molecules of a nitrogenous substance. Haemochromogen is easily oxidized in air, forming, within a certain range of *p*H, the reddish brown compound of parahaematin.

Haemochromogen, like haem, combines with carbon monoxide forming a CO-haemochromogen in which one molecule of the nitrogenous substance is replaced by one of CO, according to the following equation:

$$\text{Porph. Fe}^{\cdot\cdot}\begin{matrix}\text{N subst.}\\ \text{N subst.}\end{matrix} + \text{CO} \underset{\text{light}}{\overset{\text{dark}}{\rightleftharpoons}} \text{Porph. Fe}^{\cdot\cdot}\begin{matrix}\text{CO}\\ \text{N subst.}\end{matrix} + 1\text{ N substance.}$$

This reversible reaction is light-sensitive, proceeding in the dark towards the right and in the light towards the left. Our knowledge as to the structure of haemochromogens can be summarized as follows. The four pyrrole nuclei of the porphyrin, united by four CH≡groups, are arranged in a flat closed-ring system, the hollow space of which, in haematin, is occupied by the iron atom. Of the six covalencies of Fe four lie in the same plane and connect it with the nitrogens of the pyrrole rings, while one which lies above and the other below this plane connect it, in a haemochromogen, with the two molecules of a nitrogenous substance such as pyridine. One can see therefore that the Fe of haem will not be equally accessible to different nitrogenous substances which may react with free Fe.

Of the nitrogenous substances reacting with haem, the purine caffeine (1:3:7-trimethylxanthine) is of special interest. Purines or pyrimidines occur in nucleic acids, coenzymes (pyridine-adenine dinucleotide), the prosthetic group of enzymes (riboflavin adenine dinucleotide) and in adenylic acid. On the other hand, the iminazole ring, which occurs not only in purines but also in several other biological substances, especially in histidine, was recently suspected to be one of the links by means of which globin combines with haem to form haemoglobin. Finally, caffeine and some other purines and iminazole compounds have important pharmacological properties.

Although the reaction between caffeine and haem has twice been mentioned in the literature [D. Keilin, 1933; Barron, 1937], the nature of the compound thus formed was not properly understood nor were the reactions of other purines in this respect ever recorded.

The object of this investigation is to determine the nature of the reaction taking place between haem and other tetrapyrrolic compounds with caffeine and also with other purines and iminazole derivatives.

MATERIAL AND METHODS

Haemin used in this work was prepared from horse or ox blood by the Schalfejeff method. Caffeine was recrystallized from ethanol or from water. The reactions were followed spectroscopically, using for this purpose, according to requirements, one of the following four instruments:

(1) Microspectroscope ocular attached to a microscope which enables a rapid study of solutions in layers of different thickness examined in a test-tube, kept at an angle with the microscope stage. It also makes possible the study of mixtures in very narrow flat-bottomed tubes placed vertically above the condenser of the microscope. As these tubes require a very small amount of material, they are most suitable for the study of compounds available only in small quantities. Finally, by means of the comparative prism of the microspectroscope ocular, the absorption spectrum of one compound can be compared with that of another.

(2) Hartridge reversion spectroscope in a horizontal position, or attached to a microscope in place of an ocular, was used only for rapid determination of the exact position of absorption bands.

(3) Hilger Nutting spectrophotometer was used for more detailed study of absorption spectra in the visible region.

(4) Hilger quartz Spekker photometer was used for the study of the ultra-violet absorption spectra.

In all curves representing the absorption spectra the absorption coefficient ϵ is defined as $1/cd \ln I_0/I$, where c is the concentration of the pigment in g. mol./ml., d being the length of the tube in cm., I_0 and I the intensities of incident and transmitted light respectively.

RESULTS

Reaction of haems and derived compounds with caffeine

Reaction of caffeine and protohaem. A stock solution of alkaline protohaematin, prepared by dissolving about 12 mg. protohaemin in 50 ml. of 1% Na_2CO_3 or of 0·1 *N* NaOH, was diluted, according to requirements, either with alkali or with distilled water. $Na_2S_2O_4$ reduces the haematin to haem (two diffuse absorption bands). A little caffeine turns the reduced solution from reddish brown to a distinctly red colour, and its two diffuse bands are replaced by two much stronger bands: α, 570 mμ and β, 540 mμ. Both of these bands lie nearer the blue end of the spectrum than the corresponding bands of haem, and the intensity of the α-band is much greater than that of the β-band (Fig. 1). The γ-band is also intensified and shifts from 385 to 413 mμ. Moreover, the solution becomes very clear and does not precipitate on standing.

The minimum amount of caffeine required to complete this transformation was determined by adding known amounts of caffeine to standard solutions of haem, and comparing it spectroscopically with a solution of haem containing excess caffeine. It was thus found that the formation of the new compound requires at least 20 mol. caffeine/mol. haem.

Reaction of caffeine and haems other than protohaem. Caffeine reacts similarly with other haems such as mesohaem, haematohaem and deuterohaem. Caffeine reinforces and shifts the visible absorption bands of all these substances towards the blue end of the spectrum and prevents the precipitation of these pigments.

Reactions of caffeine and CO-protohaem. CO-protohaem is much more soluble than haem, giving a red solution with the absorption bands at 565 and 542 mμ, which has no tendency to precipitation. On the addition of caffeine to CO-protohaem the absorption bands of the latter become more intense and both bands are shifted considerably towards the red end of the spectrum (Fig. 1), their positions now being α, 590 mμ and β, 551 mμ. The solution has a tendency to become more cloudy and on standing the compound precipitates.

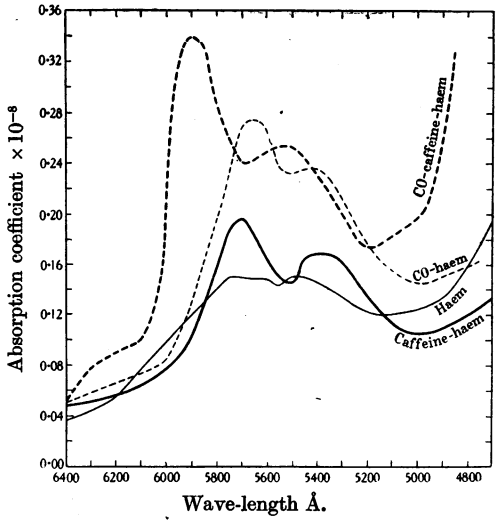


Fig. 1. Absorption spectra in the visible region of haem and CO-haem with and without caffeine.

The minimum amount of caffeine necessary for this reaction was determined in the usual way, and it was found that 1 ml. $1{\cdot}5 \times 10^{-3}M$ haem solution in 1% Na_2CO_3 requires for the formation of this compound, CO and 0·1 ml. $1{\cdot}5 \times 10^{-2}M$ caffeine, while the addition of 0·1 ml. $0{\cdot}75 \times 10^{-2}M$ caffeine gives only about 30–40% of this compound. This clearly shows that its formation requires 1 mol. of caffeine/mol. of CO-haem.

While a solution of protohaemin in 0·1 *N* NaOH, even when several days old, easily forms the caffeine CO-haem compound, the solution of haemin in 1–3% Na_2CO_3 gives this compound only when freshly prepared. Such a solution after standing for 6 hr. or overnight forms mainly or solely a typical CO-haem, the absorption spectrum of which is not modified even on the addition of a large excess of caffeine. In the absence of CO, however, it forms with caffeine the characteristic caffeine-haem compound described above. Although the old solution of haematin in Na_2CO_3 gives typical haemochromogen and CO-haemochromogen compounds with different nitrogenous substances such as pyridine, nicotine, etc., yet the effect of caffeine and CO reveals that it differs markedly from the original fresh haematin solution.

That a protohaematin solution in Na_2CO_3 does undergo some important changes on standing is also shown by two other properties: it is no longer soluble in a mixture of ether and acetic acid and it does not give haemin crystals when treated with acetic acid and NaCl. On the other hand, if the haem solution is kept in a thoroughly evacuated Thunberg tube containing an alkaline solution of $Na_2S_2O_4$ in its hollow stopper it retains all the properties of a fresh solution of haem for several weeks. Such a solution, even after 5 weeks, gives the characteristic caffeine CO-haem compound with the α-band at about 590 mμ. Clearly the irreversible changes taking place in a carbonate solution of haematin are oxidative, and are irreversible even on addition of $Na_2S_2O_4$ for the purpose of reducing haematin to haem. The caffeine-CO-haem reaction therefore represents one of the most sensitive tests for the oxidative deterioration of a haematin solution, which can be detected even a few hours after its preparation.

Reaction of caffeine and CO compounds of other haems. We have seen that such compounds as haemato-, meso- and deuterohaem react with caffeine showing characteristic changes in their absorption spectra. These haems also react with CO, forming the corresponding CO-haems. On the other hand, the addition of caffeine to these CO-haems hardly affects their absorption spectra, a fact which shows that caffeine reacts solely with the CO-protohaem prepared from protohaemin crystals.

Reaction of caffeine and haemochromogens. Pyridine deuterohaemochromogen obtained by the addition of a minimum amount of pyridine to deuterohaem forms the best material for the study of this reaction. This haemochromogen shows two very strong absorption bands: α, 550 mμ and β, 520 mμ. On the addition of an excess of caffeine the bands at first become double and then the bands of pyridine haemochromogen are gradually replaced by two more diffuse but still strong bands of the caffeine-haem compound lying at α, 560 mμ and β, 530 mμ. On the addition of more pyridine these bands rapidly disappear, and are replaced by those of the ordinary pyridine haemochromogen. These reactions give the appearance of competition between pyridine and caffeine for the haem. Similar results can be obtained by treating with caffeine the haemochromogens obtained from other haems and other nitrogenous substances. The significance of this reaction will be discussed below.

Reactions between caffeine and porphyrins. Mesoporphyrin hydrochloride, dissolved in 0·1% Na_2CO_3, gives a brownish red opalescent solution of alkaline porphyrin with a very characteristic diffuse absorp-

tion spectrum, the bands near the red end of the spectrum being the most distinct. On the addition of caffeine to this solution, the opalescence rapidly disappears, the solution becomes clear, its colour turns to salmon pink and the absorption spectrum now appears to be composed of sharp and distinct bands (Figs. 2, 3). This sharpening of the bands is

Holden considered that this reaction indicated the formation of a compound between porphyrin and globin, which suggests that 'the porphyrin residue plays a part in the formation of haemoglobin independently of the iron'.

This view was not, however, accepted by Haurowitz & Waelsch [1929], who found that although

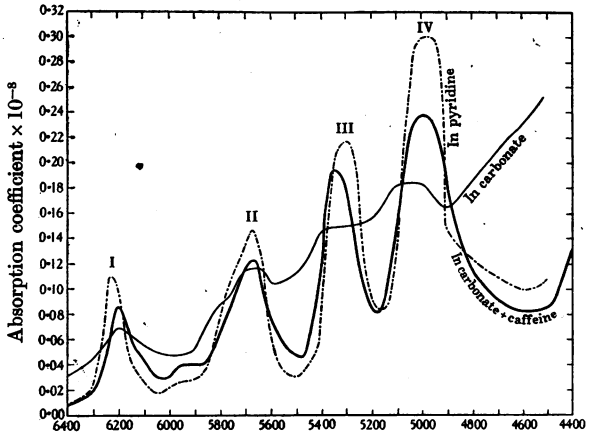


Fig. 2.

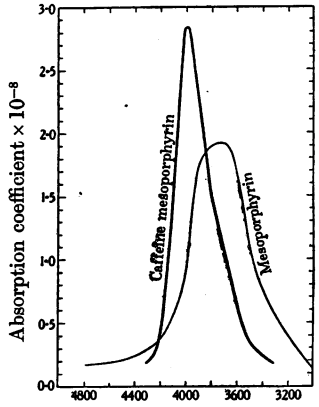


Fig. 3.

Fig. 2. Absorption spectra in the visible region of mesoporphyrin in weak carbonate solution with and without caffeine and in pyridine.

Fig. 3. Absorption spectra in ultra-violet region of mesoporphyrin in weak carbonate solution with and without caffeine.

accompanied by the displacement of the last three bands (II, III and IV) towards the blue end of the spectrum. About 20 mol. or more of caffeine are required per mol. of porphyrin to complete this change of colour and of absorption spectrum. The porphyrin thus modified remains in solution even on the addition of a little $CaCO_3$. Caffeine also affects other porphyrins such as proto- and haematoporphyrin in the same way, dispersing their colloidal solutions and changing their colour and their absorption spectra. Similar effects can, however, be obtained by treating porphyrins with solvents like pyridine and ethanol (Fig. 2), or with surface active substances such as sodium desoxycholate, sodium taurocholate, digitonin and sodium dodecyl sulphate which dissolve or disperse the aggregated molecules of porphyrin.

The reaction of porphyrins with caffeine shows a great similarity to the reaction of porphyrin with globin. Hill & Holden [1926] found that when haemato- or mesoporphyrin in dilute $NaHCO_3$ is added to a solution of native globin, the colour of the porphyrin changes from orange to pink and its absorption bands, previously diffuse, become very sharp and intense. Moreover, the solution thus obtained is no longer precipitated by $CaCO_3$. Hill & serum proteins affect the colour and the absorption bands of porphyrin in the same way as does globin, yet these proteins form no haemoglobin with haem. It is, on the other hand, possible that for the formation of haemoglobin the protein must combine both with the porphyrin and with the iron of haem, and that 'serum proteins' fail to give haemoglobin because in their native state they do not react with the iron of haem.

Reactions of caffeine with metalloporphyrins. The best material for the study of this reaction is turacin. This pigment, described for the first time by Church [1870; 1892] as a copper porphyrin compound, was considered by Fischer & Hilger [1924] to be composed of copper and uroporphyrin I. Its structure was recently reinvestigated by Rimington [1939] who identified it as a compound of copper with uroporphyrin III, the natural isomer allied to the porphyrin of haemoglobin.

Turacin is very soluble in weak alkalis giving a clear purplish red solution with two absorption bands: α, 562·5 mμ and β, 526·0 mμ. On acidifying this solution, turacin changes its colour from purplish red to orange, and the two absorption bands are shifted towards the red end of the spectrum: α, 583 mμ and β, 542 mμ. The pigment gradually

aggregates until it forms a flocculent orange precipitate. This change in colour and absorption spectrum takes place even if the precipitation of turacin is prevented by the addition of a colloidal protector such as 0·2–0·4% gum arabic. The absorption spectrum of turacin in feathers is the same as that of its acid suspension. It was clearly demonstrated [D. Keilin, 1926] that the two distinct absorption spectra of turacin are due to different degrees of dispersion of the pigment, the bands shifting towards the blue end of the spectrum as the degree of dispersion increases and towards the red end of the spectrum when the molecules of the pigment begin to aggregate. In fact, the absorption spectrum of turacin in acid ethanol and in aqueous alkaline solution is the same.

The dispersing properties of caffeine can therefore easily be tested with turacin. For this purpose a red feather of a Turaco bird is washed in ether and immersed in a very weak solution of ammonia, giving a characteristic purplish red turacin solution. On acidifying this solution with acetate buffer *p*H 4·5 the solution turns rapidly orange and the absorption bands are shifted towards the red end of the spectrum (α, 583 mμ; β, 542 mμ). If a small amount of caffeine is now added, the colour of the solution becomes purplish red and the absorption bands return to their initial position (α, 562 mμ; β, 526 mμ), although the solution remains distinctly acid. This clearly shows that caffeine disperses the aggregates of acid turacin probably by forming a complex with its porphyrin. Even an alkaline solution of turacin is affected by caffeine; this is shown by a perceptible intensification and a slight shift of its absorption bands further towards the blue end of the spectrum. Caffeine also intensifies and shifts the absorption bands of other metalloporphyrins such as manganese mesoporphyrin. It is interesting to note that although a caffeine-like effect on turacin can be obtained with solvents like ethanol and pyridine, it cannot be imitated by surface active substances or detergents such as bile salts or Na dodecyl sulphate.

Nature of reaction between caffeine and haem

We have seen above that the addition of caffeine to haem changes its colour from reddish brown to red and markedly affects its absorption spectrum by reinforcing and shifting its bands.

It is generally believed that the changes taking place in haem on the addition of a nitrogenous substance are always due to the formation of a haemochromogen compound, in other words to a direct combination of the Fe$^{\cdot\cdot}$ of haem with two molecules of this nitrogenous substance. In fact, the reaction between caffeine and haem was previously ascribed to the formation of such a caffeine-haemochromogen compound [D. Keilin, 1933; Barron, 1937]. A more detailed study of this reaction, however, does not support this view. On the contrary, it shows that caffeine reacts not with the iron but with the porphyrin of the haem, and caffeine-haem is therefore a more appropriate name for such a compound. This supposition is supported by the following considerations:

(1) Caffeine, unlike the haemochromogen-forming nitrogenous substances, even within the characteristic very limited range of *p*H, never forms a parahaematin compound with haematin.

(2) It is well known that haematin does not form a true solution even in alkali. It is always present in the form of molecular aggregates reaching a molecular weight of about 50,000 [Zeile & Gnant, 1940]. After reduction the aggregates of haem rapidly increase in size, and eventually form a precipitate. The diffuse absorption spectrum of haem is probably due to the formation of these large molecular aggregates. The clarification of the haem solution, and the reinforcement and shift of its absorption bands towards the blue end of the spectrum, caused by the addition of caffeine, can only be ascribed to the dispersion of these large molecular aggregates of haem. In fact, similar changes in haem can be observed on the addition of solvents such as ethanol, or of the previously mentioned surface active substances.

(3) That the dispersion of haem is probably due to complex formation between caffeine and the porphyrin of haem is strongly supported by the fact that caffeine reacts in a similar way with other metalloporphyrins such as turacin, as well as with free porphyrins.

(4) The effect of caffeine on haemochromogens suggests at first sight the possibility of competition between caffeine and pyridine for the Fe$^{\cdot\cdot}$ of haem. This competition is, however, an apparent one, as similar results can be obtained by using a suitable solvent or surface active substance.

Nature of reaction between caffeine and CO-haem

This remarkable effect of caffeine can be tentatively explained in two different ways:

(1) It is possible that caffeine combines directly with the Fe of CO-haem and forms CO-caffeine haemochromogen, although, as we have seen, it may not form with haem a caffeine haemochromogen. This supposition is supported by the following considerations:

(*a*) CO-haem is known to have a much greater affinity than the free haem for nitrogenous substances.

(*b*) Only one molecule of caffeine per molecule of CO-haem is required for the complete change in the absorption spectrum of the CO-haem. It is known, on the other hand, that the formation of CO-haemochromogen requires only one molecule of a nitrogenous substance per Fe atom of haem.

(*c*) The addition of caffeine to CO-protohaem shifts its absorption bands towards the red end of the spectrum and makes the compound less soluble. This is very unlike its effect on haem, on the metalloporphyrins and on porphyrins which, on the contrary, are dispersed and made more soluble. It is known, however, that while CO-haem is one of the most soluble derivatives of haematin in dilute alkali, CO-haemochromogen is a much less soluble compound.

(2) An alternative and more plausible explanation is that caffeine, as in the case of other tetrapyrrolic compounds, reacts with the porphyrin and not with the Fe of CO-haem. This is supported by the following considerations:

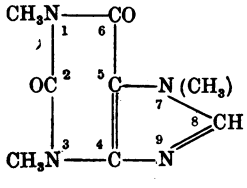
(*a*) The characteristic effect of caffeine on CO-haem is obtained only if the haem is prepared from a solution of protohaematin; it has no marked effect on the CO-compounds of other haems such as meso-, haemato-, or deuterohaem.

(*b*) Unlike CO-haemochromogen, the caffeine CO-haem compound is not affected by light.

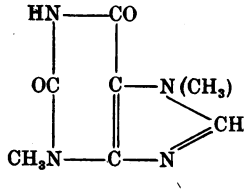
(*c*) On the addition of a solvent like ethanol, or of surface active substances such as bile salts, the characteristic absorption bands of caffeine-CO-haem are rapidly replaced by those of the ordinary CO-haem compound. Moreover, on the addition of pyridine, an ordinary CO-pyridine haemochromogen is formed.

Reaction of purines other than caffeine with tetrapyrrolic compounds

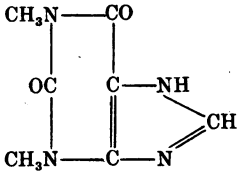
This study revealed the remarkable fact that caffeine and chlorocaffeine are the only purines which react with haem and its derivatives in



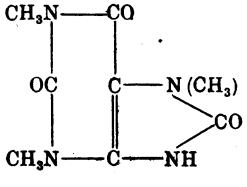
caffeine



theobromine



theophylline



1:3:7-trimethyl uric acid

the manner described above. The other purines examined in this respect were completely inert and negative results have been obtained with adenine, guanine, xanthine, hypoxanthine, paraxanthine, heteroxanthine, theophylline, theobromine, uric acid and at least twelve of its derivatives including 1:3:7-trimethyl uric acid. Yet the structures of three of these compounds, theophylline, theobromine and 1:3:7-trimethyl uric acid, closely resemble that of caffeine.

It seems that in order to react with haem or with a porphyrin, the purine must have the nitrogen atoms 1, 3 and 7 methylated; the hydrogen in position 8 may be replaced by chlorine but not by oxygen.

Effects of pyrimidine and iminazole compounds on haem and its derivatives

The three pyrimidines—thymine, cytosine and uracil—tested in the usual way, produced no effect either on different haems or on their porphyrins.

Iminazole compounds, on the other hand, are known to react with haematins and their derivatives. Küster & Koppenhöfer [1927] were the first to suggest that the iminazole ring of histidine probably represents the group by means of which the haem is attached to globin to form haemoglobin. The great affinity of 4(5)-methyl iminazole for haem with formation of haemochromogen, and for haematin with formation of parahaematin was shown by Holden & Freeman [1929]. They find it 'difficult to believe that the iminazole ring of histidine is the sole or even the chief group in proteins which combines with reduced haematin. It may well be, especially in the case of denatured globin, that some of the molecules of reduced haematin combine with two different types of nitrogen atom, one of which is in the iminazole ring'. Langenbeck [1932] obtained crystalline parahaematins of glyoxaline and 4(5)-methyl iminazole. The facts, (i) that certain iminazole compounds have such a great affinity for haematin, and (ii) that globin is so rich in histidine, suggest, according to Langenbeck, that histidine must play an important part in linking haematin to globin. Haurowitz [1935], however, pointed out that not all iminazole compounds have high affinity for haematin; histidine itself does not form a stable parahaematin compound nor on reduction does it give a haemochromogen; globin of invertebrate haemoglobins is not rich in histidine.

The comparative study of other iminazole compounds in respect of their reactions with alkaline porphyrin, haematin, haem, CO-haem and acid turacin were carried out on other purines (*v. sup.*), iminazole, iminazole 4:5-dicarboxylic acid, 1-methyl iminazole, benziminazole, 1-methyl benziminazole, histidine, histamine, ergothioneine, anserine, carnosine and pilocarpine. The results of these experi-

ments, which are shown in Table 1, can be summarized as follows:

(1) Caffeine and chlorocaffeine are the only purines which give the characteristic reactions described above. Other purines, including theobromine, theophylline and 1:3:7-trimethyl uric acid, do not react with either haematin, haem, other metalloporphyrins, or porphyrins.

histidine and β-alanine, forms with haem a typical haemochromogen but fails to react with porphyrins.

(5) The fact that globin [Hill & Holden, 1926] and serum proteins [Haurowitz & Waelsch, 1929] react with porphyrins in the same way as caffeine or pilocarpine, suggests that some of the constituents of these proteins must have certain properties in common with the 1-methyl iminazole derivative.

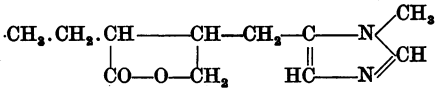
Table 1. *Reaction of different iminazole compounds with tetrapyrrolic compounds*

0 means no appreciable reaction observed.
'Caffeine effect' means dispersion, solution, reinforcement and shift of bands.

	Haematin	Haem	CO-haem	Alkaline porphyrin	Acid turacin
Caffeine and chlorocaffeine	0	Caffeine-haem	Caffeine-CO haem	Caffeine effect	Caffeine effect
Other purines	0	0	0	0	0
Iminazole	Parahaematin	Haemochromogen	CO-haemochromogen	0	0
1-Methyl iminazole	Parahaematin	Haemochromogen	CO-haemochromogen	Caffeine effect	Caffeine effect
Iminazole 4:5-carboxylic acid	Parahaematin	Haemochromogen	CO-haemochromogen	0	0
Benziminazole	0	0	0	0	0
Methyl benziminazole	0	Haemochromogen	CO-haemochromogen	0	0
Histidine	Parahaematin	Haemochromogen	CO-haemochromogen	0	0
Histamine	Parahaematin	Haemochromogen	CO-haemochromogen	0	0
Ergothioneine	0	0	0	0	0
Anserine	Parahaematin	Haemochromogen	CO-haemochromogen	0	0
Carnosine	0	Haemochromogen	CO-haemochromogen	0	0
Pilocarpine	Parahaematin	Haemochromogen	CO-haemochromogen	Caffeine effect	Caffeine effect

(2) The other iminazole compounds, unlike caffeine, combine with the Fe of haematin, of haem and of CO-haem, forming respectively parahaematin, haemochromogen and CO-haemochromogen. Histidine forms these compounds with difficulty, while ergothioneine does not seem to form them at all.

(3) Of all the iminazole compounds tested, only pilocarpine, and to a lesser degree, 1-methyl iminazole (in addition to their ability to form parahaematins, haemochromogens and CO-haemochromogens), share with caffeine the property of reacting with and dispersing the porphyrins and metalloporphyrin compounds. In the alkaloid pilocarpine, the 1-methyl iminazole ring is attached solely by its carbon 5 to another ring which can be easily separated as homopilopic acid:



Pilocarpine

(4) The fact that with porphyrin pilocarpine reacts like caffeine and with haem like 1-methyl iminazole or even pyridine, shows that the failure of caffeine and of other purines to react with the $Fe^{..}$ of haem and to give a haemochromogen compound, is probably due to the steric hindrance caused by the pyrimidine ring. It is also interesting to note that anserine, a dipeptide of methylated

However, the only iminazole compound so far known to occur in proteins is histidine which, as we have seen, does not react with porphyrins. Negative results in this respect were also obtained with other constituents of proteins such as alanine, arginine, aspartic acid, cystine, cysteine, glutamic acid, glycine, hydroxyproline, leucine, lysine, methionine, phenylalanine, proline, serine, tryptophan, tyrosine and valine.

The observation that some proteins have a very strong 'caffeine effect' on aqueous solution of alkaline porphyrin, while no known constituent of these proteins shows such an effect, can be explained in two ways:

(1) The protein may contain an additional unknown constituent with the properties of the iminazole ring of caffeine or pilocarpine.

(2) The iminazole ring of histidine, when this amino-acid forms part of a polypeptide, may differ in certain properties from that of the free amino-acid, and may resemble more the iminazole ring of caffeine and pilocarpine.

More work, however, is required before one or other of these explanations can be definitely accepted and important information as to the chemical structure of proteins and the properties of polypeptide chains may be obtained by making use in these investigations of the reactions with haematins and their derivatives.

Physico-chemical properties of caffeine and its pharmacological action

It is well known that caffeine acts on the central nervous system, the kidneys, muscles and heart. The most important pharmacological action is that on urinary excretion, but there is no general agreement as to the mechanism of caffeine diuresis: various interpretations were critically reviewed by Verney & Winton [1930] and by Smith [1937]. Ellinger, Heymann & Klein [1921] and Brühl [1929] alone have made an attempt to correlate caffeine diuresis with certain physico-chemical properties of this drug, and only the results obtained by these workers will therefore be discussed. Ellinger *et al.* found that the rate of ultrafiltration of Ringer solution, mixed with serum, through a Bechhold collodion membrane (impermeable to proteins) is increased 60% by the addition of as little as 1 in 56,000 caffeine. This is ascribed by these workers to the direct effect of caffeine on the properties of proteins, lowering their affinity for water, and not to the increased permeability of the filter membrane or glomeruli. This physico-chemical mechanism explains, according to Ellinger *et al.*, all the facts of caffeine diuresis. On the other hand, Brühl [1929] found that caffeine protects protein from precipitation by low concentrations of ethanol and increases the solubility of congo-red, and of certain amino-acids. Brühl ascribes these effects to the ability of caffeine to combine with proteins and other organic substances, and this seems to agree with some of the results obtained by other workers [Schüller, 1929; Labes & Rubenbeck, 1933]. He found also that the permeability to buffer solutions of collodion membranes impregnated with albumin or globulin, is much increased by the addition of caffeine. According to Brühl the diuretic effect of this drug is due to an increased permeability of glomeruli, a view previously expressed by Cushny & Lambie [1921]. Neither Ellinger *et al.* nor Brühl has compared the physico-chemical properties of caffeine which were observed with those of theobromine and theophylline. There is little doubt that the reactivity of caffeine and its solvent and dispersing properties discussed above are intimately connected with the physico-chemical properties of this drug which are considered by Ellinger *et al.* and by Brühl to explain the mechanism of caffeine diuresis. We have seen, on the other hand, that in its physico-chemical properties as revealed by reactions with tetrapyrrolic compounds, caffeine differs very markedly from theobromine and theophylline. If therefore these properties of caffeine have a direct bearing on its physiological action, the diuresis induced by the three purine derivatives cannot be ascribed to one and the same mechanism. All this shows that the intimate mechanism of caffeine diuresis will probably only be solved by a comparative physico-chemical and physiological study of the three diuretic purines carried out in the same way and under the same conditions.

SUMMARY

1. On the addition of caffeine to an alkaline solution of protohaem, the solution turns from reddish brown to red, and its two diffuse absorption bands are replaced by much stronger bands lying nearer the blue end of the spectrum. The solution loses its opalescence and on standing does not precipitate. A similar effect is produced by caffeine on other haem compounds such as meso-, deutero- and haematohaem.

2. Contrary to the view held by previous workers, the compound thus obtained is not a caffeine-haemochromogen. In this reaction caffeine combines, not with the Fe" of haem, but with its porphyrin as the following evidence shows: (*a*) Addition of caffeine to an aqueous alkaline solution of porphyrin changes its colour from orange to salmon pink, and greatly intensifies and shifts its absorption bands. The solution loses its opalescence and does not precipitate on standing. (*b*) Caffeine intensifies and shifts the absorption bands of metalloporphyrin compounds. It disperses and dissolves a suspension of acid turacin, the colour and the absorption spectrum of which become similar to those of an alkaline solution of this pigment, although the reaction of the solution remains acid. (*c*) About 20 mol. of caffeine per mol. of haem or porphyrin are required to produce the complete transformation. (*d*) The effect of caffeine on haems and on porphyrins can be obtained by treating these pigments with solvents, or with surface active substances such as bile salts or sodium dodecyl sulphate.

3. Caffeine reacts with CO-protohaem shifting its two absorption bands from α, 565 mμ and β, 542 mμ to 590 mμ and 551 mμ respectively. Only one molecule of caffeine per molecule of CO-protohaem is required to complete this change.

4. This effect of caffeine can be obtained with CO-protohaem prepared from a fresh solution of haematin. After standing in air for 24 hr. or less, the solution in carbonate fails to give the characteristic caffeine CO-haem compound, while the same solution, if kept anaerobically, gives this reaction even after several weeks. In carbonate solution haematin is apparently very unstable in air.

5. Caffeine gives no reaction of this nature with CO-haem compounds obtained from other haematins such as haemato-, deutero- and mesohaematin.

6. Two possible explanations as to the mechanism of the reaction between caffeine and CO-haem are proposed and discussed.

7. Caffeine and chlorocaffeine are the only purines which react as described with haem, CO-protohaem, porphyrins and metalloporphyrins. No other purine, out of about 20 tested, including theobromine, theophylline and 1:3:7-trimethyl uric acid, gives any of these reactions.

8. The fact that chlorocaffeine behaves in this respect like caffeine, shows that the H in position 8 can be replaced by Cl but not by O_2.

9. Of all the iminazole compounds tested, only pilocarpine, and to a lesser degree 1-methyl iminazole, react with the porphyrin in the same way as caffeine; but, unlike caffeine, with haematin, haem and CO-haem, they give parahaematin, haemochromogen and CO-haemochromogen compounds respectively.

10. The fact that globin and certain serum proteins react with porphyrins in the same manner as caffeine, while none of their amino-acid constituents, including histidine, gives this reaction, suggests that (*a*) either these proteins contain an additional, not yet isolated, constituent having these properties, or (*b*) that the iminazole ring of histidine, when the latter is incorporated in a polypeptide chain, may have some of the properties of the iminazole ring of caffeine and of pilocarpine.

11. The bearing of all these properties of caffeine on the mechanism of caffeine diuresis is discussed.

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The Action of Proteolytic Enzymes on Fibrinogen Solutions

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It has been recognized for some years that a proteolytic enzyme system may be concerned with blood coagulation. Wright [1915] noted that blood coagulation could be inhibited by the addition of trypsin, and Douglas & Colebrook [1916] confirmed this observation, but demonstrated that smaller quantities of trypsin had the effect of *shortening* the clotting time.

Further studies of the effect of trypsin on blood coagulation have been reported by Tyson & West [1937], Eagle & Harris [1937], and Mellanby & Pratt [1938]. Though differences are evident between these investigations all the authors agree that trypsin in sufficient amounts prevents coagulation, and assume that the fibrinogen is destroyed. More recently, Tagnon, Davidson & Taylor [1942] have demonstrated the presence in chloroform-treated serum of a proteolytic enzyme which appears to be derived from the plasma globulins. This enzyme, like trypsin, prevents coagulation in large amounts, while in smaller amounts it hastens coagulation with subsequent digestion of the clot.

The work reported here is concerned mainly with the effect of pepsin on fibrinogen solutions and on plasma at *p*H values of 5·0–5·6. This limited *p*H range is necessary because at values below 5·0 fibrinogen tends to precipitate, and at values higher than 5·6 pepsin is inactivated.