slicing had been devised and used for some years, Majno & Bunker (1957) described a more elaborate apparatus for slicing in which a blade is fixed horizontally on a stand and the tissue moved by hand across and above the blade. This apparatus also yields such slices. Two models of the apparatus have been examined, one with the mounted microtome blade described by Majno & Bunker (1957) and another employing a strip of razor blading. With both, the cutting process could not be watched; the slice was more difficult to transfer, and an aqueous medium was always required in cutting. [*Added August* (1960). In the apparatus of Hultin, Arrhenius, Löw & Magee (1960), also, the slice is not visible during cutting and is again subject to shearing forces from the whole width of a cutting blade.] The guide described here allows the progress of the cutting to be seen and thus to be better controlled; with the bow cutter it allows slicing without added aqueous fluids. For these reasons the simple recessed guide used as described above appears the more satisfactory and versatile instrument.

SUMMARY

1. The tissue chopper previously described has undergone development and has been further applied.

2. For slicing tissues, the preparation and use of a simple guide is described.

3. A bow cutter is described for use with the guide when cutting tissue in the absence of added aqueous fluid.

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Use of Carbon Monoxide for Detecting Denaturation in Haemoproteins

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Through the work of Hoppe-Seyler (1889) it became known that haemoglobin forms a compound with carbon monoxide. Hill (1926, 1930) observed that combination with carbon monoxide takes place also with the isolated prosthetic group of haemoglobin in the form either of haem or of its pyridine compound pyridine haemochromogen. The reactivity towards carbon monoxide observed both with haemoglobin and its prosthetic group is by no means characteristic of haem compounds in general. On the contrary, many haem compounds, among them the important class of intracellular respiratory pigments known as cytochromes, appear as a rule to be inert towards carbon monoxide. Keilin, in his pioneer investigations on these respiratory pigments, encountered only one exception to this rule in animal tissues, namely cytochrome a_3 (Keilin & Hartree, 1939).

As soon, however, as their native structure is disorganized by exposure to alkali, or by other influences, cytochromes as a rule can become reactive towards carbon monoxide. It thus appears that the reaction with carbon monoxide in the neutral pH range provides a convenient means of detecting denaturation. In numerous publications, however, investigators failed to report the behaviour of their preparations towards carbon monoxide, leaving it open to doubt whether their

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observations were made with native cytochromes, or with partly or totally denatured compounds. This is the more surprising since the test had already been in use for years (see, for example, Slater, 1949, 1958; Strittmatter & Ball, 1952).

Though numerous investigations involving carbon monoxide–haem compounds, beginning with the observations of Hoppe-Seyler and including the recent studies of Smith (1959), have contributed much to our knowledge of haem–carbon monoxide compounds, we are unaware of any systematic survey of the use of carbon monoxide for detecting denaturation of haemoproteins. Correlation of data reported in the literature is not easy. Earlier experiments were carried out with the help of gasometric methods or by visual microspectroscopy, with the result that the spectral changes in the ultraviolet and the near-ultraviolet range of the spectrum were often neglected.

Moreover, spectroscopic observations were made under insufficiently defined conditions, as it was not at first realized that haem compounds are susceptible to oxidative degradation in a sodium carbonate medium and that they are strongly affected by changes in pH, the nature of the solvent and the degree of dispersion of the pigment (Keilin, 1926; Keilin, 1943, 1949; Shack & Clark, 1947; Smith, 1959).

It was considered desirable to repeat earlier experiments by means of a quartz spectrophotometer under better defined and comparable conditions. The spectra changes occurring in a carbon monoxide test are illustrated on a simplified model of a denatured haemoprotein. An example of the application of the test to a haemoprotein preparation isolated from heart muscle will be described.

MATERIALS AND METHODS

Haemin. The method of Labbe & Nishida (1957) was used for purification of haemin from ox blood.

Haemoglobin. A sample prepared from ox erythrocytes was provided by Dr D. H. Deul in this laboratory. The preparation contained much methaemoglobin.

Cytochrome c. A preparation from horse heart isolated by the method of Keilin & Hartree (1945) was used.

Carbon monoxide. CO was generated by dropping pure formic acid into warm conc. H_2SO_4. The gas was collected in a gasometer above concentrated aqueous $(NH_4)_2SO_4$.

Spectroscopy. For preliminary qualitative and semi-quantitative spectroscopic examinations, a Zeiss–Winkel microspectroscope was used.

Absorption measurements were carried out with a Zeiss spectrophotometer (PMQ-II) with absorption cells with a light path of 1 cm. For low extinctions 2 cm. cells were used and the values recalculated for a light path of 1 cm. Readings were taken at 1 mμ intervals in the critical parts of the spectrum and at 5 or 10 mμ intervals elsewhere. The wavelength-scale calibration of the spectrophotometer was checked against two emission bands of the hydrogen lamp.

Procedure for the carbon monoxide test. To 5 ml. of the solution containing the haem compound a few grains of $Na_2S_2O_4$ were added. The sample, contained in a wide test tube, was saturated with CO by passing through a finely divided stream of the gas for 5 min. The sample was then transferred to an absorption cell and a few additional grains of $Na_2S_2O_4$ were stirred in to bring the final concentration to about 1%. To prevent reoxidation and escape of CO, the sample was covered with a layer of liquid paraffin, about 4 mm. high, and the absorption cell closed with a rubber lid. The complete spectrum was measured within 10 min. The initial absorption values were unchanged after 30 min.

Difference spectra. Bubbling CO through the solutions usually increased the background absorption of the samples to a variable extent by changing the state of dispersion of the pigments. For the sake of uniformity the difference spectra were calculated on the assumption that at 540 mμ the extinction of the reduced and the CO-reduced compounds were equal.

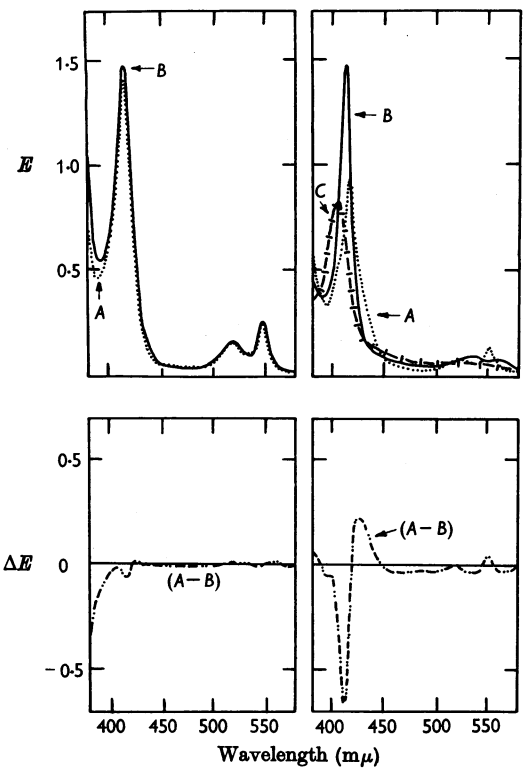

Fig. 1 Fig. 2

Fig. 1. Effect of CO on the spectrum of native cytochrome *c*. 9·16 μM-Cytochrome *c* in 0·05 M-phosphate, pH 7·4. *A* ···, $+Na_2S_2O_4$; *B*, —, $+Na_2S_2O_4+CO$; —·—, difference spectrum (*A* − *B*), adjusted so that $\Delta E_{540\ m\mu}$ is 0.

Fig. 2. Effect of CO on the spectrum of denatured cytochrome *c*. 9·16 μM-Cytochrome *c* in 0·05 M-phosphate, pH 7·4. *A*, ···, $+Na_2S_2O_4$; *B*, —, $+Na_2S_2O_4+CO$; —·—, difference spectrum (*A* − *B*), adjusted so that $\Delta_{540\ m\mu} = 0$; *C*, −|−|, oxidized with $K_3Fe(CN)_6$.

RESULTS

Fig. 1 shows the absorption spectrum of a cytochrome *c* preparation, Fig. 2 the spectrum of the same preparation after extensive denaturation by treatment with 0·6 N-KOH for 24 hr. at 32° and subsequent neutralization. With the extensively denatured preparation the α- and β-bands at 550 and 520 mμ are flattened by treatment with CO and replaced by rather diffuse bands at 562 and 533 mμ respectively, whereas the Soret band is sharpened and shifted from 416 to 412 mμ. The slight increase in the Soret band brought about by CO (Fig. 1) is no doubt due to contamination with denatured cytochrome *c*, which is usually present in small amounts in cytochrome *c* prepared by the above-mentioned procedure. Fig. 2 includes the spectrum of the same denatured cytochrome *c* preparation after treatment with $K_3Fe(CN)_6$. There is no indication that the CO compound of the reduced cytochrome *c* was contaminated by oxidized cytochrome *c*. However, the resemblances between the flattened α- and β-bands of the oxidized form and the CO compound of the reduced pigment make it advisable to be sure of complete reduction by adding excess of $Na_2S_2O_4$.

Haemoglobin was denatured by treatment with alkali until it had lost its capacity to combine with O_2. The spectrum (curve *A*, Fig. 3) showed the typical sharp α- and β-bands of a haemochromogen. Addition of CO (curve *B*, Fig. 3) caused a considerable weakening and flattening of these bands, whereas the Soret band was increased in intensity. Curve *C*, Fig. 3, shows for comparison the spectrum of the CO compound of native haemoglobin. This is similar to that of the CO compound of denatured haemoglobin, but the absorption is greater.

In the next set of experiments an artificial protein-free haemochromogen served as a simple model of a denatured haemoprotein. The denatured

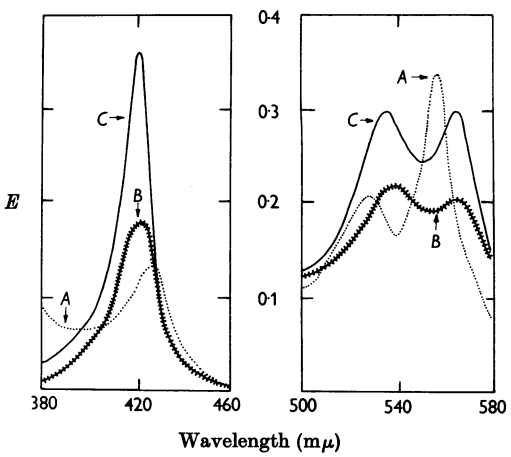

Fig. 3. Effect of CO on the spectrum of native and denatured haemoglobin. Haemoglobin was denatured by treatment with 0·2 N-NaOH for 1·5 hr. at 20°. The spectrum was measured in 0·1 M-phosphate, pH 7·4, with 20 μM-haemoglobin in visible range and 4 μM in u.v. range. *A*, ..., denatured haemoglobin + $Na_2S_2O_4$; *B*, -|-|, denatured haemoglobin + $Na_2S_2O_4$ + CO; *C*, —, native haemoglobin + CO.

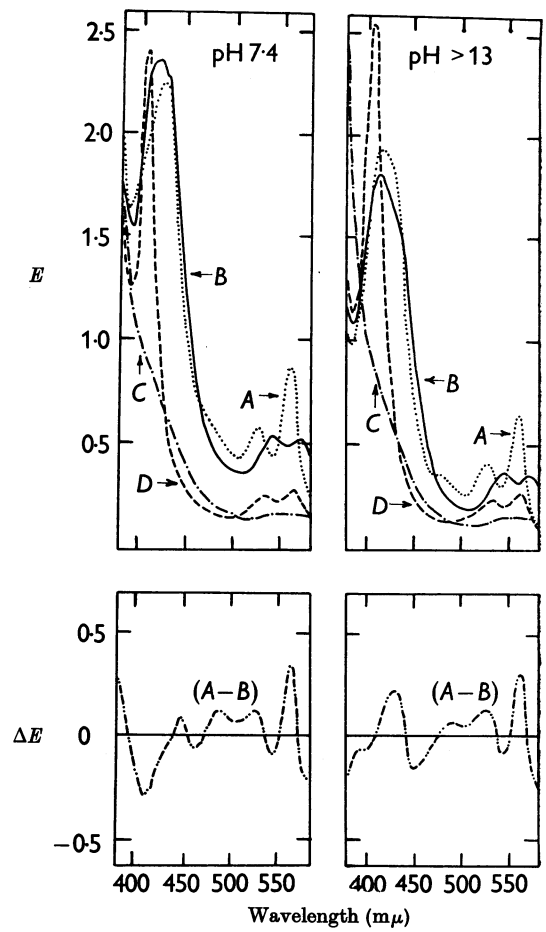

Fig. 4 Fig. 5

Fig. 4. Effect of CO, at neutral pH, on spectrum of pyridine haemochromogen in the presence of a low concentration of pyridine. All solutions contained 2 mM-haem, 0·1 M-phosphate, pH 7·4, and 1% of $Na_2S_2O_4$. *A*, ···, +0·075% (w/v) of pyridine; *B*, —, 0·075% (w/v) of pyridine + CO; *C*, —·—, no addition; *D*, - - -, +CO; —·—, difference spectrum (*A* − *B*).

Fig. 5. Effect of CO, at high pH, on spectrum of pyridine haemochromogen in the presence of a low concentration of pyridine. 0·1 M-Phosphate buffer, pH 7·4, was replaced by 0·1 N-NaOH. Other conditions and *A*, *B*, *C* and *D* were as in Fig. 4.

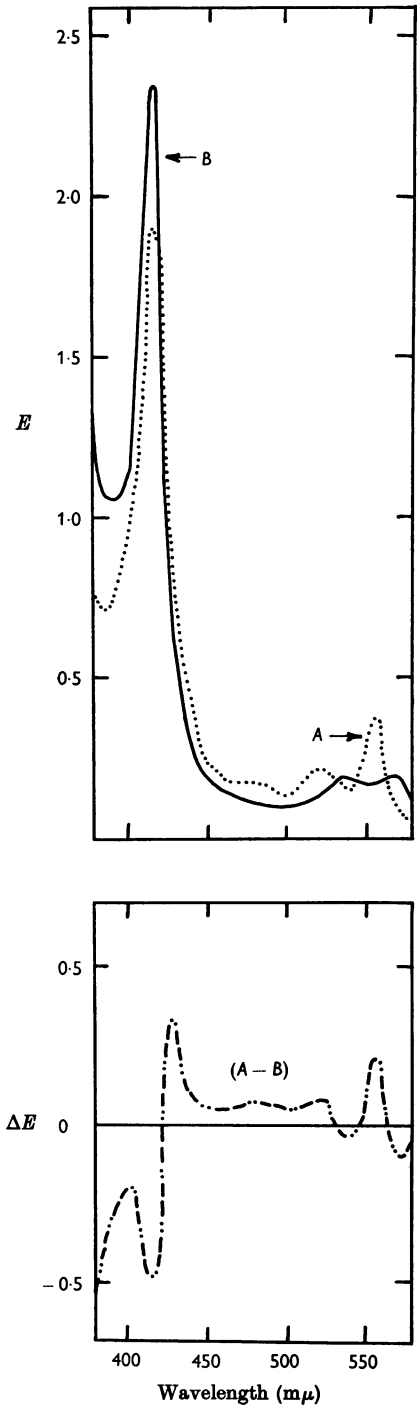

Fig. 6. Effect of CO on spectrum of pyridine haemochromogen in the presence of a high concentration of pyridine. Both solutions contained 1 mM-haem, 0·1 N-NaOH, 20% (w/v) of pyridine and 1% of $Na_2S_2O_4$. *A*, ···, no further addition; *B*, —, +CO; —·—, difference spectrum (*A* − *B*).

protein linked to the haem iron was replaced by pyridine. The CO test was carried out under two sets of conditions. In the first, the pyridine concentration was kept comparatively low, in order to obtain a maximum effect with CO, by favouring the right-hand side of the equilibrium equation

$$\text{pyr–Hm–pyr} + \text{CO} \rightleftharpoons \text{pyr–Hm–CO} + \text{pyr},$$

where pyridine is represented by pyr and haem by Hm, the hyphens denoting attachment to the haem iron. Figs. 4 and 5 show the effect of CO at nearly neutral and at strongly alkaline pH, respectively. In the second set of experiments a high pyridine concentration (20%) corresponding to the standard conditions for preparing pyridine haemochromogens was used (cf. Hartree, 1955). Even here a marked change of the spectrum resulted (Fig. 6). A flattening effect of CO on the α- and β-bands is evident in all three cases, irrespective of the variation in pH and pyridine concentration. The Soret bands behave differently. With 20% of pyridine there is an increase in absorption with no appreciable shift in the position of the band (Fig. 6). With the low pyridine concentration less

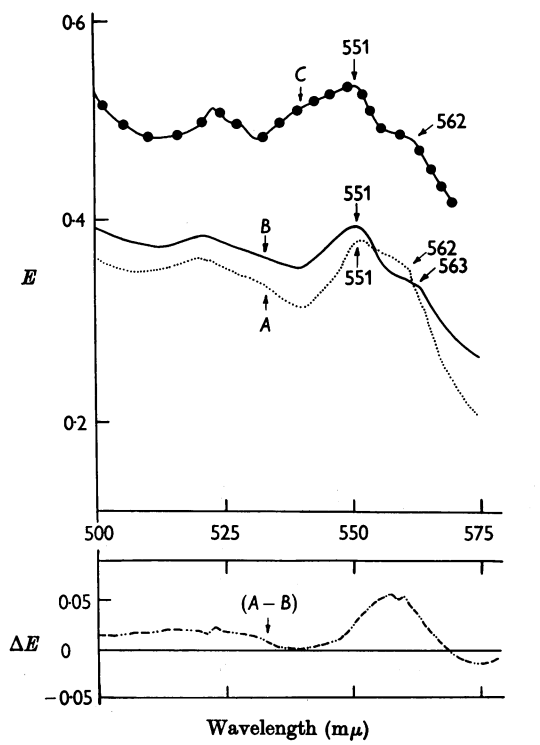

Fig. 7. Effect of CO on spectrum of a cytochrome *b* preparation. The preparation was solubilized with 1% of deoxycholate in 0·1 M-phosphate buffer, pH 7·4. *A*, ···, $+Na_2S_2O_4$; *B*, —, $+Na_2S_2O_4$+CO; *C*, —•—, +sodium succinate; —·—, difference spectrum (*A* − *B*).

sharp Soret bands are obtained; the shapes of the long-wavelength slopes suggest the presence of other components (Figs. 4, 5). Yet whereas at strongly alkaline pH a small decrease in absorption takes place, at pH 7·4 the Soret band slightly increases. For comparison the spectra of reduced haem and haem–CO in the absence of pyridine are also shown in Figs. 4 and 5.

Fig. 7 illustrates an application of the CO test in investigations concerning cytochrome *b*. In an attempt at purifying this cytochrome from ox heart according to a fractionation procedure of Bernstein & Wainio (1958), a preparation was obtained, which upon addition of succinate showed two weak absorption bands at 551 and 562 mμ, respectively. Upon addition of $Na_2S_2O_4$ a broad and strong absorption band appeared in the range 551–560 mμ. The difference in the spectra obtained with succinate and $Na_2S_2O_4$, respectively, appears to correspond to the amount of denatured haemoprotein present. The preparation which we obtained probably contained both cytochrome c_1 and cytochrome *b* with most of the cytochrome *b* in an enzymically inactive, denatured state. Treatment in the presence of $Na_2S_2O_4$ with CO resulted in a spectrum that showed a striking similarity to that obtained with succinate. The difference spectrum of Fig. 7 exhibits a peak at 558–560 mμ, corresponding presumably to the α-band of denatured cytochrome *b*.

DISCUSSION

Table 1 summarizes the main spectral changes in our experiments. Wherever carbon monoxide compounds were formed with denatured haemochromogens or with pyridine haemochromogens, two kinds of changes in the absorption bands took place. (i) A shift of the position of the bands towards the longer wavelengths was generally observed with α- and β-bands; a shift towards the shorter wavelengths was observed with the γ-bands, except in the experiment involving the presence of 20% of pyridine, in which no shift was found with the γ-band. (ii) In most cases there occurred also an appreciable change in the height of the bands. The bands in the visible part of the spectrum decreased, whereas the γ-bands usually increased in height and sharpness. In Table 1 an increase or decrease in a band is indicated merely as such without giving numerical values; such values could bear full significance only if the extent of denaturation of the haemoprotein had been estimated by a different method and could be quantitatively correlated. The absorption spectra and the difference spectra in Figs. 1–7 nevertheless give a measure of the flattening or sharpening of the bands due to reaction with carbon monoxide. In the experiments in which pyridine haemochromogen served as a model of a denatured haemoprotein, some additional minor peaks or shoulders were observed which are not included in Table 1, e.g. the shoulder at 430 mμ of the pyridine haemochromogen–CO compound depicted in Fig. 4, which no doubt indicates residual pyridine haemochromogen. The appearance of these shoulders is in keeping with the observations of other investigators. When Smith (1959) attempted to prepare pyr–Hm–CO by applying carbon monoxide to pyr–Hm–pyr, residual unconverted pyr–Hm–pyr invariably persisted. He was, however, able to prepare the pyr–Hm–CO compound in full yield by a different sequence of reactions, i.e. by preparing first

Table 1. *Effect of combination with carbon monoxide on absorption bands of haem compounds*

		Absorption bands in reduced state						Change in extinction	
		Before CO treatment			After CO treatment				
	pH	α	β	γ	α	β	γ	Visible bands†	Soret band†
Cytochrome *c* (native)	7·4	550	520	415	550	520	415	–	+
Cytochrome *c* (denatured)	7·4	550	520	416	562	532	412	–	+
Pyridine haemochromogen (0·075% of pyridine)	7·4	560	527	426	572	540	416	–	+
Pyridine haemochromogen (0·075% of pyridine)	13	560	526	416	570	542	412	–	+
Pyridine haemochromogen (20% of pyridine)	13	555	523	418	568	538	418	–	+
Haemoglobin (native)	7·4	555	.	430	566	538	420	.	.
Haemoglobin (denatured)	7·4	560	.	425	568	540	422	–	+
Cytochrome *b* preparation	7·4	558–563	.	428*	563‡ (551)	.	419*	–	.
Haem	7·4	540–580	.	.	564	532	407	.	.
Haem	13	540–580	.	.	564	532	406	.	.

* Value obtained with a different cytochrome *b* preparation.
† +, Increase in *E*; −, decrease in *E*.
‡ Shoulder.

CO–Hm–H_2O and then adding pyridine. In our experiments we did not attempt to achieve a 100% conversion into the carbon monoxide compound, as it was our main purpose to imitate as closely as possible, on a simpler model, the procedure applied in the test for denatured haemoproteins. The measurements were also deliberately restricted to a spectral range where dithionite does not interfere with the spectrum and therefore can conveniently serve as a reducing agent. In the spectral range below 390 mμ rather elaborate methods of reduction have to be resorted to, which do not commend themselves for a simple routine test.

The experiment illustrated in Fig. 7 shows the use of both an enzymic method and the carbon monoxide test for estimating the degree of denaturation in a cytochrome *b* preparation. Disregarding the roughly constant difference in extinctions of curves *B* and *C*, which may be attributed to a change in background absorption (compare 'Difference spectra' in the Materials and Methods section), the agreement of the two methods appears reasonable. A comparatively simple enzymic test was possible in this particular case, owing to the fact that the crude cytochrome *b* preparation still contained some succinic dehydrogenase. Further purification of the cytochrome *b* would be most likely to lead to a preparation no longer reducible by succinate. At such a stage, failure of the absorption bands to appear on addition of succinate would no longer be conclusive. It might be due either to absence of succinic dehydrogenase or to a total denaturation of the cytochrome *b*. An enzymic method gives the most direct evidence of the native state of a cytochrome and, wherever practicable, deserves preference. The carbon monoxide test, on the other hand, has the advantage that it can also be applied when conditions for an enzymic test are not known or where they cannot easily be achieved.

The effect of combination with carbon monoxide on the spectra of haem and of pyridine haemochromogen, contained in an aqueous medium, appears to be connected with, if not due to, a depolymerization of the previously dimeric haem compound (Shack & Clark, 1947; Smith, 1959). It is a subject for further investigation to show if one could account in an analogous way for the spectral changes observed upon addition of carbon monoxide to denatured haemoprotein.

SUMMARY

1. The effects of carbon monoxide on the spectra of some native and denatured haem compounds are compared.

2. Pyridine haemochromogen was used as a model of a denatured haemoprotein reacting with carbon monoxide.

3. The usefulness of carbon monoxide as a method of detecting denaturation of haem proteins is discussed.

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