Reaction of Ethyl Isocyanide with Haemocyanin

MANY compounds have been tested for their ability to react with the oxygen-binding site of haemocyanin and it has been found that thiourea[1] and thiocyanate[2] cause the expulsion of oxygen from *Helix pomatia* haemocyanin with a consequent decrease in the absorption of the copper bands at 346 and 570 mμ. This effect was almost completely reversed by dialysis or by treatment with an anion exchange resin. A report on the exchange reaction of ethyl isocyanide with oxygen in oxyhaemoglobin[3] encouraged us to investigate the effect of this compound on haemocyanin. Ethyl isocyanide does not remove copper from haemocyanin, but it does cause a decrease in the copper bands, which presumably indicates the expulsion of oxygen.

Haemocyanin was obtained from the haemolymph of the whelk, *Murex trunculus*, either by the method of Bannister *et al.*[4], or by passing the haemolymph through a column (80 × 2·4 cm) of 'Sephadex *G*-200' equilibrated with 0·05 molar *tris*-hydrochloric acid, *p*H 7·0, containing 0·1 molar potassium chloride. Ethyl isocyanide was synthesized by the method of Jackson and McKusick[5]. Copper was estimated by the method of Peterson and Bollier[6], and protein was estimated from the absorbance at 280 mμ in 0·1 molar borate buffer, *p*H 9·2 at which *p*H the error caused by light scattering is minimal[7].

The addition of ethyl isocyanide in concentrations from 0·5 to 50 mmolar to the haemocyanin caused a very rapid decrease in the copper bands at 346 and 570 mμ. The reaction was almost complete in less than 1 min at room temperature, and thereafter there was a slow drop in absorbance during a period of hours. Fig. 1 shows the relationship between concentration of ethyl isocyanide and absorbance at 346 mμ after 3 min (that is when the reaction is at least 95 per cent complete). The slow decrease in absorbance over longer periods may be partly caused by the fact that ethyl isocyanide is gradually hydrolysed in aqueous solution with the production of cyanide, which removes the copper from haemocyanin.

That ethyl isocyanide does not remove the copper atoms from haemocyanin is illustrated by the following experiment. To 3 ml. of haemocyanin (34·5 mg) in 0·1 molar phosphate buffer, *p*H 7·5, was added 0·05 ml. ethyl isocyanide (0·7 mmoles). The absorbance at 570 mμ was noted continuously for 10 min, during which time it dropped by 44 per cent. The solution was then passed through a column (40 × 2·4 cm) of 'Sephadex *G*-25' equilibrated with 0·1 molar *tris*-hydrochloric acid, *p*H 8·3. The protein-containing fractions of the eluate were pooled and analysed for copper in duplicate. The original haemocyanin solution was analysed for copper at the same time. Aliquots of the column effluent and of the original haemocyanin were diluted in 0·1 molar borate buffer, *p*H 9·2, and their absorption spectra were recorded in a Beckman '*DB*' spectrophotometer. The copper content of the original haemocyanin was 0·240 per cent, and that of the haemocyanin treated with ethyl isocyanide was 0·250 per cent. From the absorption spectra, the ratios $E_{346m\mu}/E_{280m\mu}$ and $E_{570m\mu}/E_{280m\mu}$ were calculated. The values of these ratios were, for haemocyanin, 0·282 and 0·022; and for ethyl isocyanide-treated haemocyanin, 0·263 and 0·019, respectively. It was possible to regenerate the copper bands simply by bubbling oxygen through the mixture of ethyl isocyanide and haemocyanin. Ethyl isocyanide was added to haemocyanin in 0·1 molar phosphate buffer at *p*H 7·5. After 5 min the absorbance at 346 mμ had decreased to 45 per cent of its original value. Pure oxygen was then bubbled through the mixture for 10 min. After 5 min the absorbance had risen to 76 per cent, and after 10 min to 80 per cent of its original value.

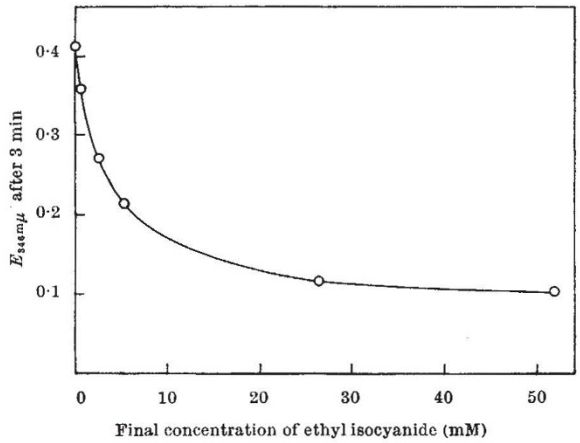

Fig. 1. Relationship between final concentration of ethyl isocyanide and absorbance of *Murex trunculus* haemocyanin at 346 mμ after 3 min. To 2·5 ml. of haemocyanin in 0·1 molar borate buffer, *p*H 9·2 (protein concentration, 1 mg/ml.), was added ethyl isocyanide as an emulsion in the same buffer, to the final concentration indicated. The absorbance at 346 mμ was noted continuously in a 1 cm cell. The emulsion of ethyl isocyanide also absorbed at 346 mμ, and so the same amount of ethyl isocyanide was added to 2·5 ml. of borate buffer in the reference cuvette.

The exchange reaction of ethyl isocyanide with oxygen in oxyhaemocyanin should prove useful in studies of the mode of binding of oxygen by haemocyanin. Ethyl isocyanide displaces the oxygen more effectively than thiourea and thiocyanate, and the reaction is easily reversible. Rombauts and Lontie[1,2] found that at the greatest concentrations of thiourea and thiocyanate they used, only about 10 per cent and 23 per cent respectively of the original absorbance of the copper bands remained. We have found that in optimal conditions (0·5 mg/ml. of haemocyanin in 0·1 molar borate buffer, *p*H 9·2; molar ratio of ethyl isocyanide to copper, 4,000) the absorbance of haemocyanin at 346 mμ can be reduced to less than 1 per cent its original value by treatment with ethyl isocyanide.

E. J. WOOD
W. H. BANNISTER

Department of Physiology,
Royal University of Malta,
Valletta,
Malta.

Received March 16, 1967.

[1] Rombauts, W., and Lontie, R., *Arch. Intern. Physiol.*, **68**, 230 (1960).
[2] Rombauts, W., and Lontie, R., *Arch. Intern. Physiol.*, **68**, 695 (1960).
[3] Sumita, N., Okazaki, T., Shukuya, R., and Kaziro, K., *J. Biochem.* (Tokyo), **55**, 188 (1964).
[4] Bannister, W. H., Bannister, J. V., and Micallef, H., *Experientia*, **22**, 626 (1966).
[5] Jackson, H. L., and McKusick, B. C., *Org. Synth.*, **35**, 62 (1955).
[6] Peterson, R. E., and Bollier, M. E., *Anal. Chem.*, **27**, 1195 (1955).
[7] Heirwegh, K., Borginon, H., and Lontie, R., *Biochim. Biophys. Acta*, **48**, 517 (1961).

Uptake of Selenite labelled with Selenium-75 by Human Leucocytes *in vitro*

PREVIOUS work (reviewed in ref. 1) has shown that after the injection of selenite labelled with selenium-75 (SeO_3^{--}) into animals radioactivity is incorporated into the proteins of various tissues. Recent studies in our laboratory[2] have demonstrated that in humans selenite is rapidly taken up by the liver, kidneys and by malignant tumours. Analyses of both normal and tumour tissues have indicated that the selenium is bound to tissue protein by a covalent linkage. We have investigated the mechanism by which inorganic selenium is taken up by human cells.

Plasma rich in leucocytes was obtained from normal human venous blood by the method of Moorhead *et al.*[3] using phytohaemagglutinin. Sterility was maintained throughout the procedure. To 4 ml. of this plasma was added 8 ml. of tissue culture medium, *TC*-199 (Difco)[4], and 1 ml. of 0·9 per cent sodium chloride con-