[4] H. L. KORNBERG AND W. E. PATEY, *Biochim. Biophys. Acta*, 25 (1957) 189.
[5] M. F. UTTER AND H. G. WOOD, *Advances in Enzymol.*, 12 (1951) 41.
[6] J. W. DAVIS, V. H. CHELDELIN, B. E. CHRISTENSEN AND C. H. WANG, *Biochim. Biophys. Acta*, 21 (1956) 101.
[7] H. L. KORNBERG, *Biochem. J.*, (in the press).
[8] H. L. KORNBERG AND N. B. MADSEN, *Biochim. Biophys. Acta*, 24 (1957) 651.
[9] H. L. KORNBERG AND H. A. KREBS, *Nature*, 179 (1957) 988.
[10] S. OCHOA, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. I, Academic Press Inc., New York, 1955, p. 739.

Received April 11th, 1957

Cross linking of collagen by S- and N-mustards

When di-(2-chloroethyl) sulphide (mustard gas, H) is applied to the skin, about 12% of the amount which penetrates remains bound to skin tissue. The extent of the resultant vesication has been shown to be approximately proportional to the amount of H fixed[1].

The present work was therefore carried out to examine the reaction between mustard gas and related S-compounds (S-mustards) and N-compounds (N-mustards) and collagen, the major protein component of skin. PIRIE[2] investigated the reaction between H and ox-corneal collagen and found the reaction product differed in certain aspects from untreated collagen. Swelling in acid and alkaline solution was much reduced and solubility in boiling water and digestibility by proteolytic enzymes were negligible. These results suggest that H might be acting as a cross-linking agent, stabilising the structure of the collagen molecule. On the other hand, ALEXANDER, FOX, STACEY AND SMITH[3] found that neither H nor the N-mustard, di-(2-chloroethyl) methylamine (HN2), brought about any obvious cross-linking of the fibrous protein of wool. In the present study an attempt was made to obtain further evidence for the cross-linking of collagen by some S- and N-mustards and other cross-linking agents. A study was therefore made of the effect of these various substances on the shrinkage temperature, T_s, of collagen. THEIS[4] has defined T_s as "the specific point at which the increasing disruptive tendencies exceed the diminishing cohesive forces, thus making the shrinkage temperature actually a measure of the structural stability of collagen expressed in temperature units". Thus, if cross-linking has occurred and if measurements of T_s are made under similar conditions of pH and ionic strength[4], an increase in T_s would be expected.

Standard hide powder (B.D.H.) was used in these experiments and treatment with the various reagents was carried out as follows:

(a) With the N-mustards, 2,4-dinitrofluorobenzene (FDNB), 1,3-difluoro-4,6-dinitrobenzene (DFDNB) and tri-acryl formal, 150 mg of hide powder were suspended in 10 ml of 0.25 *M* phosphate buffer, pH 7.4, containing the reagent (10 mmoles) and the suspension agitated for 16 h at 37°. The fall in pH during the incubation was never greater than 0.5 of a unit.

(b) In the case of the S-mustard, however, the phosphate buffer employed in (a) was too weak to maintain the pH during treatment and a much higher concentration of buffer ion was obviously necessary. Since S-mustards react readily with anions[5] it is undesirable to use high concentrations of buffer, and another method of treatment was adopted. The hide powder was suspended in 10 ml distilled water, 0.1 *N* NaOH was added as required to maintain pH between 6.8 and 8.2 (phenol red) and the reaction allowed to continue till the pH stopped decreasing (about 1.5 h). Tests using HN2 under these conditions gave similar results to those obtained with assay method (a), and consequently the two procedures (a) and (b) are strictly comparable.

In order to keep conditions of pH and ionic strength the same when determining T_s, the fibres were spun down hard after treatment with the agent and the supernatant discarded. The fibres were resuspended in 0.025 *M* phosphate buffer, pH 7.4, and spun down again. This process was repeated 6 times and finally the fibres were suspended in a small volume of buffer. Measurements of T_s were carried out by the micro-technique described by BORASKY AND NUTTING[6]. The temperature at which shrinkage occurred was read to the nearest 0.5°. It was observed, as reported by these authors, that different samples of the same group of fibres gave values of T_s varying by as much as 3°. Observations were therefore made on at least 10 groups of fibres from each sample and the mean and standard deviation calculated.

The effects of the various treatments on the T_s of hide powder are shown in Table I. The main finding was the fact that treatment with a multifunctional S- or N-mustard brought about a considerable rise in T_s, whereas treatment with the corresponding mono-functional compounds either brought about a much smaller increase in T_s or did not raise it significantly. This result is most easily explained on the assumption that the multifunctional mustards have brought

TABLE I

T_s OF HIDE POWDER COLLAGEN FOLLOWING TREATMENT WITH VARIOUS REAGENTS

Name of reagent	*No. of functional groups*	*Amount present in reaction mixture (mmoles)*	T_s °C ± S.D.	*Change in T_s due to treatment* °C
—	—	—	56.5 ± 0.5	—
2-Chloroethyl diethylamine	1	1	57.0 ± 2.5	–
2-Chloroethyl diethylamine	1	3	56.0 ± 1.0	–
2-Chloroethyl di-*iso*propylamine	1	1	57.0 ± 1.0	–
Di-(2-chloroethyl) methylamine	2	1	79.0 ± 2.0	+ 22.5
Tri-(2-chloroethyl) methylamine	3	1	86.0 ± 1.5	+ 32.5
Ethyl 2-bromoethyl sulphide	1	1	59.0 ± 1.0	+ 2.5
Benzyl 2-bromoethyl sulphide	1	1	62.5 ± 1.5	+ 6.0
Benzyl 2-bromoethyl sulphide	1	2	68.0 ± 0.5	+ 11.5
Di-(2-chloroethyl) sulphide	2	0.5	71 ± 0.5	+ 14
Di-(2-chloroethyl) sulphide	2	1.0	78.5 ± 1.0	+ 23
FDNB	1	1	62.0 ± 1.5	+ 5.5
DFDNB	2	1	97.5 ± 1.5	+ 21.0
Tri-acryl formal	3	Satd. soln. $\ll$ 1 mmole	89.0 ± 1.0	+ 32.5

about some degree of cross-linking in collagen. DFDNB[7] and tri-acryl formal, recognized cross-linking agents, also increased the T_s of hide powder.

The two monofunctional compounds which produced slight increases in T_s were benzyl 2-bromoethyl sulphide and FDNB. In these particular cases the increased T_s may be due to secondary valency forces between the bound benzyl and nitrophenyl groups and charged groups of collagen. It is of interest in this connection that ALEXANDER *et al.*[3] found that FDNB produced effects similar to cross-linking in wool fibres. Tri-acryl formal, which acts as a cross-linking agent by virtue of its activated double-bond systems, also raised the T_s of hide powder very significantly despite its low solubility.

The pH stabilities of the links between the various reagents and collagen which are responsible for the observed increases in T_s were examined at pH 1.29, 4.0, 7.5 and 10.9, by suspending samples of the treated fibres in 0.1 *N* HCl or in buffers of the appropriate pH for 16 h at 37° and noting any resulting changes in T_s. The bonds between reagents and protein appeared to be stable over a wide pH range, except in the case of H-treated fibres where incubation at pH 10.9 lowered the T_s 10°. This fact suggests that ester linkages may play a part in cross-links formed by this reagent. This finding is of interest in view of our previous report[8], where it was observed that 50% of H which reacts with collagen is bound to carboxyl groups and also that H can esterify glycine, whereas HN2 reacted less readily with glycine and the ester formed appeared to be very unstable. The final T_s of the H-treated fibres after alkaline incubation was still 10° above the normal value, indicating that other types of linkages are also involved.

I am indebted to Dr. P. ALEXANDER, the Chester Beatty Institute for Cancer Research, for a gift of tri-acryl formal and to Miss J. FAIRALL for technical assistance.

G. A. J. GOODLAD*

Ministry of Supply, Chemical Defence Experimental Establishment, Porton, Wiltshire (England)

1 H. CULLUMBINE, *Nature*, 159 (1947) 151.
2 A. PIRIE, *Biochem. J.*, 41 (1947) 185.
3 P. ALEXANDER, M. FOX, K. A. STACEY AND L. F. SMITH, *Biochem. J.*, 52 (1952) 179.
4 E. R. THEIS, *Trans. Faraday Soc.*, 42B (1946) 244.
5 A. G. OGSTON, *Trans. Faraday Soc.*, 44 (1948) 45.
6 R. BORASKY AND C. NUTTING, *J. Am. Leather Chemists' Assoc.*, 44 (1949) 830.
7 H. ZAHN, *Kolloid-Z.*, 121 (1951) 39.
8 G. A. J. GOODLAD, *Biochim. Biophys. Acta*, 24 (1957) 645.

Received March 16th, 1957

* Present address: Department of Biochemistry, University of Glasgow, Scotland.