# The Mechanism of the Reaction Between Di-(2-chloroethyl) Sulphone (Mustard-Gas Sulphone) and Amino Acids

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It has been demonstrated that both di-(2-chloroethyl) sulphone (mustard-gas sulphone, $HO_2$) and divinyl sulphone react with proteins and with the amino groups of α-amino acids to give derivatives of 1:4-thiazan 1:1-dioxide, the products obtained in each case being the same, whichever sulphone is used (Boursnell, Francis & Wormall, 1946*a*, *c*). A. H. Ford-Moore and A. H. Ford-Moore & A. G. Lidstone (personal communications) have also shown that $HO_2$ in alkaline solution loses HCl to give 2-chloroethyl vinyl sulphone, and then divinyl sulphone, and have suggested that this reaction precedes the reaction with amino groups. The finding that $HO_2$, which had previously been treated at 37° with bicarbonate buffer at pH 7·4 until most of its chlorine had become ionized, reacts with amino acids more rapidly than does $HO_2$, but less rapidly than does divinyl sulphone (Boursnell *et al.* 1946*a*), also lends support to this view.

The nucleophilic substitution reaction between a chloroethyl group and an amino group involves first the formation of a carbonium ion:

$$X\cdot CH_2\cdot CH_2\cdot Cl \rightarrow X\cdot CH_2\cdot CH_2^+ + Cl^-.$$

The carbonium ion may then react in one of two ways, either by direct condensation with the amino group and expulsion of $H^+$ from the latter:

$$X\cdot CH_2\cdot CH_2^+ + HNR_2 \rightarrow X\cdot CH_2\cdot CH_2\cdot NR_2 + H^+ \quad (1)$$

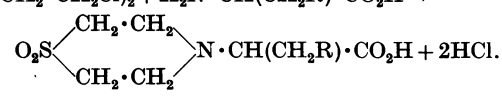
or by liberation of $H^+$ with formation of a vinyl group, followed by addition of the amino compound to the ethylenic bond:

$$X\cdot CH_2\cdot CH_2^+ \rightarrow X\cdot CH{:}CH_2 + H^+$$
$$X\cdot CH{:}CH_2 + HNR_2 \rightarrow X\cdot CH_2\cdot CH_2\cdot NR_2. \quad (2)$$

If mechanism (1) is correct, both hydrogen atoms on the carbon atom in the β-position to the nitrogen in the product would be derived from the chloroethyl group. If mechanism (2) is correct, one of these hydrogen atoms would be derived from the chloroethyl group and one from the amino group.

In order to determine to what extent a preliminary formation of vinyl groups occurs in the reaction between $HO_2$ and amino acids we have prepared the condensation products of two amino acids, α-alanine and β-phenylalanine, with $HO_2$ labelled with deuterium on all eight of its hydrogen atoms. The over-all reaction can be represented:

$$O_2S(CH_2\cdot CH_2Cl)_2 + H_2N\cdot CH(CH_2R)\cdot CO_2H \rightarrow$$




If this reaction follows mechanism (1) exclusively, the products should each contain eight labelled hydrogen atoms/molecule, but if mechanism (2) operates exclusively, only six. A result between six and eight would indicate simultaneous operation of both mechanisms.

## EXPERIMENTAL

*'Deutero'-mustard-gas sulphone.* This was prepared from deutero-mustard gas (Boursnell, Francis & Wormall, 1946*b*) by oxidation with $H_2O_2$–acetic acid as described by Francis, Mulligan & Wormall (1955). The product was diluted with exactly 49 times its weight of ordinary $HO_2$ and recrystallized from ethanol.

*$HO_2$–amino acid derivatives.* These were prepared essentially as described previously (Boursnell *et al.* 1946*a*). Alanine (0·01 mole) and β-phenylalanine (0·0075 mole) were each treated with an equimolar amount of the deutero-$HO_2$ in the presence of three equivalents of M-$NaHCO_3$. The mixtures were shaken at 37° for 3 hr. and left overnight at room temperature, the pH being maintained at about 7·5 (phenol red internal indicator) by the addition of drops of N-NaOH as required. The alanine derivative was isolated *via* the copper salt (Boursnell *et al.* 1946*a*) and the phenylalanine derivative by acidification with HCl. The alanine derivative was recrystallized from 50% (v/v) ethanol (17 ml.) and the phenylalanine derivative from boiling water (300 ml.).

For reasons given below the possibility was considered that the phenylalanine–sulphone derivative, like the corresponding glycine derivative, contained a firmly bound molecule of water of crystallization (Boursnell *et al.* 1946*a*). Analysis of the preparation (Found: C, 55·0; H, 6·1; N, 4·9; S, 11·2. $C_{13}H_{17}NSO_4$ requires C, 55·1; H, 6·05; N, 4·95; S, 11·3%) and the fact that there was no significant loss of weight when it was dried at 100° showed, however, that the phenylalanine–sulphone was free from water of crystallization.

*Determination of deuterium.* Samples (30–50 mg.) of the two amino acid derivatives and of the deutero-$HO_2$ (diluted and recrystallized with an equal weight of ordinary $HO_2$) were covered with freshly ignited CuO and burnt in dry $O_2$ in a silica combustion tube filled with CuO (wire, form M.A.R.).

Table 1. *Comparison of deuterium contents of deuterodi-(2-chloroethyl) sulphone and its condensation products with amino acids*

| Compound | Mol. formula | Atom % excess of deuterium* | No. of 'sulphone hydrogens'/molecule† |
|---|---|---|---|
| Di-(2-chloroethyl) sulphone | $C_4H_8SO_2Cl_2$ | 1·84±0·021 (3) 1·80–1·88 | 8 |
| 'Alanine sulphone' (α-thiazan-4-ylpropionic acid 1:1-dioxide) | $C_7H_{13}NSO_4$ | 0·87±0·011 (7) 0·83–0·91 | 6·2 |
| 'Phenylalanine sulphone' (β-phenyl-α-thiazan-4-ylpropionic acid 1:1-dioxide) | $C_{13}H_{17}NSO_4$ | 0·58±0·0074 (8) 0·56–0·61 | 5·4 |

* The mean values, S.E., number of determinations (in parentheses) and extreme range of values are given in each case.

† Calculated by multiplying the ratio (atom % excess of D in compound)/(atom % excess of D in $HO_2$) by the number of H atoms/molecule of compound.

The water formed was collected in a trap cooled in solid $CO_2$–ethanol and redistilled (cf. Graff & Rittenberg, 1952). The water was reduced to $H_2$ by heating with Zn dust at 400° (Dubbs, 1953), and the deuterium content of the gas was determined on a Consolidated-Nier Isotope Ratio Mass Spectrometer, calibrated with $H_2$ samples of known deuterium content.

## RESULTS

The results of the deuterium determinations on the $HO_2$ and the two $HO_2$-amino acid compounds are shown in Table 1. It is always difficult to obtain closely concordant results in repeated deuterium determinations on an organic compound, owing to the relatively large errors introduced by very small amounts of moisture contamination occurring at any stage during the manipulations, but the figures given for atom percentage excess deuterium are the means of a number of determinations, the spread of individual results and the standard error being given in the table in each case.

## DISCUSSION

The results of the deuterium determinations of the two amino acid–$HO_2$ condensation products preclude the possibility of any significant reaction proceeding via a direct nucleophilic substitution mechanism. The result with the alanine derivative is just within the limits of experimental error for a mechanism proceeding exclusively via the formation of two vinyl groups. With the phenylalanine derivative the result is lower than that required by this mechanism by an amount slightly greater than can be accounted for by experimental error in the determinations. Since the presence of water of crystallization in the compound is precluded by the elementary analyses, it appears probable that there was a slight loss of the deuterium label during this particular preparation. The loss of deuterium from compounds of this type by an exchange reaction seems unlikely, but it may be significant that this compound, unlike the alanine–sulphone, was at one stage of its preparation in contact with a strongly acid medium. Any exchange of deuterium which did occur must, however, have been very small, otherwise the deuterium content would have been considerably lower. It is highly improbable that an exchange reaction could have accounted for the result in both cases being so close to six, if, in fact, significantly more than six of the hydrogen atoms were derived from the sulphone.

It therefore appears that, at least with these two amino acids, the reaction proceeds exclusively via the formation of two vinyl groups, followed by addition of the amine to form the cyclic compound. Since $HO_2$ reacts with proteins chiefly as a result of condensation with the free amino groups of the protein (Banks, Boursnell, Francis, Hopwood & Wormall, 1946), it seems logical to conclude that in this reaction also the condensation occurs only after the $HO_2$ has been converted into divinyl sulphone.

## SUMMARY

1. α-Thiazan-4-ylpropionic acid 1:1-dioxide and β-phenyl-α-thiazan-4-ylpropionic acid 1:1-dioxide labelled with deuterium in the thiazan ring have been prepared by treating alanine and β-phenylalanine respectively with di-(2-chlorotetradeuteroethyl) sulphone in aqueous solution at 37° and pH 7–8.

2. Deuterium determinations on these two compounds show that in each case six hydrogen atoms/molecule carry a deuterium label.

3. From this result, and other evidence, it is concluded that the reaction between di-(2-chloroethyl)

sulphone and the amino groups of α-amino acids proceeds exclusively *via* the formation of two vinyl groups in the sulphone, followed by the addition of the amine to the ethylenic bonds.

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# The Quantitative Estimation of Pyruvic and α-Oxoglutaric Acids by Paper Chromatography in Blood, Urine and Cerebrospinal Fluid

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It is now recognized that the estimation of pyruvate by the Friedemann & Haugen method (1943) carries with it an element of uncertainty owing to the non-specific nature of this technique. This lack of specificity may well be unimportant for estimations in human blood and cerebrospinal fluid, in which, under normal conditions, other α-keto acids are present in small or negligible amounts, but if a clearer insight is to be gained into the disturbances of the Krebs cycle in disease a more specific determination of the individual α-keto acids is required. This is especially true if α-oxoglutaric and other α-keto acids are present in significant amounts as, for example, in the blood of certain animals such as the rat, in certain tissues and in urine. A specific procedure involving the formation of the 2:4-dinitrophenylhydrazones of the α-keto acids and their separation by paper chromatography was devised by Cavallini, Frontali & Toschi (1949*a*, *b*), and since then a number of modifications of their method have been published. These methods, however, with few exceptions have been tedious and time-consuming, and in few is it possible to assess their accuracy. The present method has been developed from that of El Hawary & Thompson (1953) and has given satisfactory results over three years. The number of extractions has been greatly reduced, and the need for evaporating the extracted hydrazones to dryness eliminated. The addition of ethanol to the ethyl acetate used for extracting the hydrazones has further shortened the procedure by greatly reducing interference due to emulsions, which are particularly apt to develop during blood determinations.

## METHOD

*Reagents.* 2:4-Dinitrophenylhydrazine, 0·2% in 2N-HCl; ethanol, absolute; ethyl acetate (A.R.); ammonium hydroxide, 2·5N; metaphosphoric acid, 5 and 10% (w/v) freshly prepared from a stock 50% solution; solvent mixture: *n*-butanol–ethanol–N-$NH_3$ soln. (13:2:5, by vol.); sodium hydroxide–sodium carbonate solution, freshly prepared by mixing equal parts of 2·5N-NaOH (A.R.) and 10% (w/v) $Na_2CO_3$ (A.R.).

*Blood.* Immediately after withdrawal 5–7 ml. of blood is mixed with 20 ml. of 5% metaphosphoric acid in a weighed tube. After a few minutes the mixture is centrifuged.

*Urine.* If the specific gravity is 1·02 or over, 3 ml. of urine is added to 13 ml. of 5% metaphosphoric acid. Below this specific gravity, increasing quantities of urine are added to decreasing amounts of more concentrated metaphosphoric acid, the amount of urine depending on the specific gravity, and the final concentration of the acid being not less than 4%. If the urine contains protein this must first be precipitated and removed by centrifuging.

*Cerebrospinal fluid.* A volume (3 ml.) is mixed with 14 ml. of 5% metaphosphoric acid, and centrifuged.

*Preparation and extraction of keto acid hydrazones.* A volume (2 ml.) of the 2:4-dinitrophenylhydrazine in 2N-HCl is added to 16 ml. of the centrifugate or, with protein-free urine, of the clear solution, and the mixture is incubated for 20 min. at 38°. The mixture is then shaken in a 30 ml. separating funnel with 5 ml. of ethyl acetate and 5 ml. of ethanol for 0·75 min., and the layers are allowed to separate. The aqueous phase is re-extracted successively with 5 ml. and then with 1 ml. of ethyl acetate. With blood filtrates some degree of emulsification usually interferes with the complete separation of the two phases during the second and third extractions. If this occurs the emulsion is added to the first ethyl acetate extract and centrifuged at 2000 rev./min. for 1–2 min. The aqueous layer is returned to the separating funnel and extracted for the third time. The contents of the