Electrophoretic separation of the reduction products of the S-sulphonated insulin chains*

Splitting of insulin into its component chains is usually carried out by sulphitolytic cleavage of the —S—S— bonds in the presence of sodium tetrathionate. For recombination studies[1,2] or for experiments on the biological behaviour of the separated chains[3], their S-sulpho derivatives [$A(S\cdot SO_3^-)_4$ and $B(S\cdot SO_3^-)_2$, where A and B indicate respectively glycyl and phenylalanyl chains] are further converted into the reactive sulphydryl form by treatment with an excess of a thiol, usually mercaptoethanol or thioglycolic acid. If the reduction is incomplete, intermediate derivatives containing both $—S\cdot SO_3^-$ and —SH groups are likely to be present in the reaction mixture. From the S-sulpho-A chain one may expect three possible intermediates: $A(S\cdot SO_3^-)_3SH$, $A(S\cdot SO_3^-)_2(SH)_2$ and $A(S\cdot SO_3^-)(SH)_3$; and from the S-sulpho-B chain: $B(S\cdot SO_3^-)(SH)$. Furthermore, the completely reduced forms, $A(SH)_4$ and $B(SH)_2$ will also be present among the reaction products.

The identification of the completely S-sulphonated A and B chains is usually carried out by paper electrophoresis in a 8 *M* urea–3.3 *M* acetic acid buffer, pH 3.2. We have found that the intermediate products can be successfully separated using the same procedure; therefore this reliable and simple tool can be used not only to check the purity of the reduced forms of the chains but also to study the kinetics of the reduction reaction.

The experimental work was carried out starting from ^{125}I-labelled completely S-sulphonated A and B chains which were prepared from ^{125}I-insulin according to the procedure given by PRUITT and associates[3].

The incomplete reduction was carried out by dissolving 2.5 mg of each chain in 0.5 ml of a solution of 8 *M* urea and freshly distilled thioglycolic acid (1 *M*), keeping the reaction mixture at 25° for 60 min. Electrophoresis was carried out in a small chamber (Elvi model No. 69) in which the paper strip is placed horizontally; the support is Whatman 3MM paper. The buffer was prepared by mixing 800 ml of 10 *M* urea (in deionized water) and 200 ml of glacial acetic acid, to give a final pH 3.2.

Runs were made at a potential gradient of about 7 V/cm and at a current of 1.5 mA. After 16 h running time, the strips were removed, dried at 60° and developed by autoradiography (Ferrania 3N films).

The separation and identification of the reduction products resulting from the reaction between ^{125}I-labelled completely S-sulphonated A chain and thioglycolic acid was carried out as follows: 25 μl samples, taken from the reaction mixture, were analyzed by electrophoresis; autoradiography of the paper strips revealed five spots, all but one migrating towards the anode. A distinct tailing effect was observed when the runs were carried out at room temperature.

When electrophoresis was carried out in a cooled chamber at +4°, no tailing was observed and five symmetrical and very well separated spots were found (Fig. 1a). The faster moving component (spot No. 1 in Fig. 1a) is the completely sulphonated A chain. The identity of the remaining spots could be deduced by evaluating the total charge at pH 3.2 of each of the possible components[4].

The A chain contains one terminal amino group (charge + 1 unit), two glutamic

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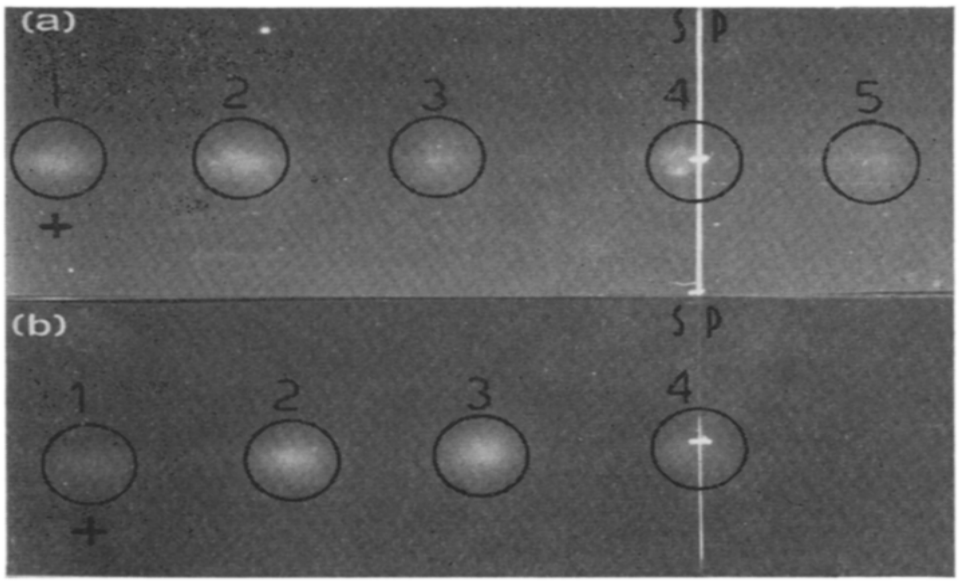

Fig. 1. Electrophoretic patterns (autoradiography) of the reduction products of the S-sulphonated A chain. (a) = Reduction of $A(S \cdot SO_3)_4$ labelled with ^{125}I; (b) = reduction of $A(^{35}S \cdot SO_3)_4$. For designations, see Table I. SP = Starting point.

acid residues ($pK = 4$, charge negligible), one terminal carboxyl group ($pK = 3$, charge $-1/2$ unit) and four S-sulphonated groups (charge of -4 units). Therefore the charge of the completely sulphonated A chain at pH 3.2 is evaluated as $-3\,1/2$ units; the reduced components will have lower negative charge values: $(A(S \cdot SO_3^-)_3\text{-}SH = -2\,1/2$; $A(S \cdot SO_3^-)_2(SH)_2 = -1\,1/2$; $A(S \cdot SO_3^-)(SH)_3 = -1/2$; or a positive value: $A(SH)_4 = +1/2$). However the identity of each spot was confirmed by double-tracer experiments. The S-sulpho A chain, labelled with ^{125}I and ^{35}S was prepared by the sulphitolytic cleavage of ^{125}I-labelled insulin using ^{35}S-labelled sodium sulphite. The resulting completely S-sulphonated chains were therefore labelled with ^{35}S in the $S \cdot SO_3^-$ group (*i.e.* $^{35}S \cdot SO_3^-$). The double-labelled A chain was reduced with thioglycolic acid and a sample of the reaction mixture was submitted to paper electrophoresis. The ^{125}I activity of each spot was directly measured on the paper strip by means of a γ-ray spectrometer. The strip was then cut in small pieces, each corresponding to a spot; each piece of paper was introduced into a glass vial together with 1 ml of 1 *M* sodium sulphite solution at pH 7; the vials were sealed, heated at 80° in a water bath for 1–2 h, then cooled at +4° and opened. The solution was transferred to a small beaker, diluted to 10 ml, and oxidation to sulphate was carried out with 1 *N* iodine. Sulphate was precipitated as the barium salt and counted in the form of a "thick layer", in a gas-flow counter. Usually no contamination of the samples by ^{125}I was found; however, all samples were checked by γ-ray counting.

From the ^{125}I activity measurements the relative amount of A chain in each spot was calculated; taking as a standard of known composition the spot of fully S-sulphonated A chain, the ^{35}S activity corresponding to four $-S \cdot SO_3^-$ groups per chain unit was calculated and used to evaluate the number of $-S \cdot SO_3^-$ groups per unit chain in each spot. The results, which are summarized in Table I, show a good agreement with the expected values, confirming the identity of each spot.

No ^{35}S activity was found in the spot corresponding to the component moving

TABLE I

NUMBER OF —S·SO_3^- GROUPS CONTAINED PER CHAIN UNIT IN EACH SPOT OF FIG. 1, AS EVALUATED BY DOUBLETRACER (^{125}I AND ^{35}S) EXPERIMENTS

*Spot No.**		*1*	*2*	*3*	*4*	*5*
Composition**		$A(SSO_3)_4$	$A(SSO_3)_3SH$	$A(SSO_3)_2(SH)_2$	$A(SSO_3)(SH)_3$	$A(SH)_4$
—S·SO_3 groups per chain unit	expected***	4	3	2	1	0
	found	—	2.91	2.10	0.95	0.01

* The numbers 1–5 refer to Fig. 1a.
** The composition is deduced on the basis of the calculation of the total charge carried by each component (see text).
*** —S·SO_3 groups per unit chain as can be expected from the composition given above.

towards the cathode (spot No. 5 in Fig. 1a), which therefore corresponds to the completely reduced form of the A chain ($A(SH)_4$).

The incomplete reduction of the ^{125}I-labelled and completely sulphonated B chain gave three electrophoretic components as shown in Fig. 2a. Here again the identity of each spot could be deduced by the calculation of the total charge carried by the different components[4].

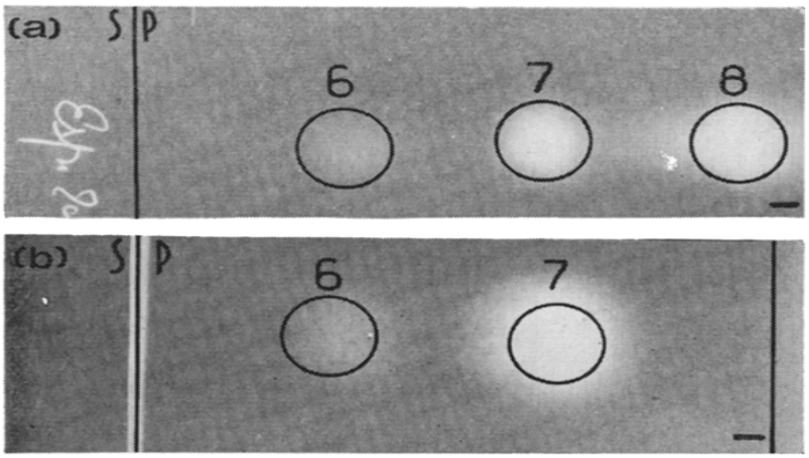

Fig. 2. Electrophoretic patterns (autoradiography) of the reduction products of the S-sulphonated B chain. (a) = Reduction of $B(S\cdot SO_3)_2$ labelled with ^{125}I; (b) = reduction of $B(^{35}S\cdot SO_3)_2$. SP = Starting point.

The completely S-sulphonated B chain contains one terminal amino group, one lysine residue, one arginine residue, two histidine residues (+5 charge units), one terminal carboxyl group (charge $-1/2$ unit) and two S-sulphonate groups (charge of —2 units). The charge of the S-sulpho B chain is therefore $+2\frac{1}{2}$ units (spot No. 6 in Fig. 2). The reduced components will have higher positive charge values. However, the faster moving spot (spot No. 8 in Fig. 2a) disappeared when the reduction experiment was repeated starting from ^{35}S-labelled completely S-sulphonated B chain; it therefore corresponds to the completely reduced B chain ($B(SH)_2$) which is unlabelled in the ^{35}S experiments.

Using as starting material S-sulphonated B chain labelled with both ^{125}I and ^{35}S, it was confirmed that the intermediate spot of Fig. 2 (spot No. 7) corresponds to a component containing one $—S \cdot SO_3^-$ group per chain unit.

The possibility that oxidative reactions of the —SH groups involving polymerization of the components might occur during the electrophoresis was checked. The relative mobilities of the different components, as evaluated by taking the spots of the completely S-sulphonated chains as a mobility standard, were found to be unchanged when electrophoresis was carried out in a 8 *M* urea, 3.3 *M* acetic acid buffer containing 0.1 *M* thioglycolic acid to prevent oxidation[4].

Further confirmation that oxidation occurs to no appreciable extent during electrophoresis was given by experiments in which the reaction products, formed by separately reducing ^{125}I-labelled S-sulphonated A and B chains, were mixed and samples of the mixture were analyzed. It was found that all components of the mixture showed a relative mobility very close to that which they showed when the derivatives of the A chain and the B chain were analyzed separately. Furthermore, the relative amount of each component was found to be in excellent agreement with that predicted on the basis of the separate analysis of the original reaction mixtures; it is likely that polymer formation would have resulted in a poor reproducibility. Here again the temperature at which the electrophoresis is carried out was found to be a critical factor. At room temperature tailing effects were observed, whereas at 4° an excellent resolution of the different components was obtained.

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