# STUDIES ON TETANUS TOXIN AND TOXOID

## III.* SEDIMENTATION OF TOXIN AND DERIVATIVES OBTAINED BY SULPHITE AND ALDEHYDE TREATMENTS

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*Summary*

Tetanus toxin prepared from toxic filtrates of *Clostridium tetani* cultures has a weight-average molecular weight of 176,000±5,000 and exhibits no detectable heterogeneity with regard to size and shape. No marked change in sedimentation properties is observed on varying pH and temperature over a selected range, but the sedimentation coefficient increases on decreasing the ionic strength.

Weight-average molecular weight determinations are used to show that the addition of sulphite ions results in partial cleavage of disulphide bonds linking subunits of the toxin. Sulphite modification does not alter the flocculation properties of the protein, but results in almost complete loss of toxicity. The findings are used to implicate a disulphide bond either at the toxic site or structurally placed to maintain the integrity of the site: this accessible bond appears distinct from that responsible for linking the subunits. Separate evidence shows that sulphydryl groups are not directly involved in the toxic site.

Reaction of the toxin with the aldehydes formaldehyde, glyoxal, and glutaraldehyde is shown by sedimentation velocity analysis to induce marked aggregation of the protein.

## I. Introduction

Sedimentation velocity studies on tetanus toxin (Pillemer *et al.* 1948; Largier 1956; Raynaud and Turpin 1956) have shown that the sedimentation coefficient of the major component determined in different environments varies in the range 5·2–7·6 S. The only available estimate of the molecular weight of the ~ 7 S material is 145,000 (Largier and Joubert 1956); the diffusion coefficient used in the estimation was found by the Lamm scale method on a sample whose homogeneity was unchecked and thus the value is unreliable. The addition of various *S*-nucleophilic bases (Parker and Kharasch 1959) and sulphydryl reagents effected the partial change of the ~ 7 S toxin, known to contain disulphide and sulphydryl groups (Bizzini *et al.* 1963), to slower sedimenting species (Raynaud, Turpin, and Bizzini 1960). The implication was that at least one disulphide bond was important in determining the structural integrity of the unit, the cleavage of the bond(s) resulting in conformational changes of the toxin or dissociation of the protein into subunits or both.

In a previous communication (Dawson and Mauritzen 1967), the isolation of tetanus toxin from toxic filtrates of *Clostridium tetani* cultures was reported. The

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method employed fractionation of samples by gradient elution on DEAE-cellulose columns and resulted in a product similar in toxicity to samples prepared by other and more elaborate procedures (Pillemer *et al.* 1948; Largier 1956; Raynaud and Turpin 1956). In this study, the preparation is shown to be homogeneous with respect to molecular size and the dependence of its sedimentation coefficient on environmental parameters is examined. Estimation of weight-average molecular weights and toxicity levels in the presence and absence of sulphite ions permits comment on the effect of cleavage of structurally important disulphide bonds. The sedimentation velocity properties of toxin treated with formaldehyde, glyoxal, and glutaraldehyde are also reported. In contrast to the effect of the addition of sulphite ions, it is shown that treatment with aldehydes leads to aggregation.

## II. Materials and Methods

Tetanus toxin was isolated from toxic filtrates of *C. tetani* cultures by the chromatographic method reported previously (Dawson and Mauritzen 1967). The material was stored in a freeze-dried form, since lyophylization proved to have no detectable effect on the sedimentation velocity properties of the toxin. It has been shown previously that freeze drying does not affect markedly either the flocculation or the lethal properties of the protein (Dawson and Mauritzen 1967).

Aldehyde treatment involved dissolution of the toxin in 0·1M phosphate buffer, pH 7·0, and introduction of the aldehyde either directly or by dialysis. The concentrations of aldehyde and protein employed are given in the text, together with other conditions for modification of the toxin.

Buffers were prepared from analytical grade materials; pH values of buffers were determined at 20°C using a Radiometer (model TTT1C) pH-meter. Toxin solutions were dialysed for at least 3 days against buffer at 4°C prior to examination in the ultracentrifuge.

Sedimentation velocity experiments were performed in a Spinco model E ultracentrifuge, employing schlieren optics and an angular velocity of 59,780 r.p.m. The temperature was recorded with the RTIC unit. In general, sedimentation coefficients were calculated from the rate of movement of the maximum ordinate of schlieren peaks, and corrected to 20°C in water to give $S_{20,w}$ values. This correction involved the partial specific volume of the protein (Svedberg and Pedersen 1940), which was estimated as 0·74 from its amino acid composition (Dawson and Mauritzen 1967). Interpretation of sedimentation velocity data on sulphite-treated toxin necessitated the evaluation of the weight-average sedimentation coefficient, $\bar{S}_{20,w}$, from the rate of movement of the square root of the second moment of the entire schlieren patterns (Goldberg 1953).

The schlieren patterns obtained by sedimenting a 0·95% tetanus toxin sample at 20°C in 0·1M phosphate buffer, pH 7·0, were subjected to a detailed analysis designed to test the homogeneity of the major component. The mathematical analysis for boundary spreading, presented by Fujita (1956) and discussed in detail by Baldwin (1957), was employed.

The Archibald method (Archibald 1947) was used to evaluate the weight-average molecular weights in samples of toxin and sulphite-treated toxin. Only values for the observed refractive index gradient at the meniscus, $(dn/dx)_m$, were employed to calculate the required ratio $(dn/dx)_m/x_m c_m \omega^2$ (where $x_m$ is the distance from the centre of rotation, $c_m$ is the concentration of the solute at the meniscus in appropriate refractometric units, and $\omega$ is the angular velocity). The quantity $c_m$ was obtained by the method of Klainer and Kegeles (1955), employing a synthetic-boundary cell.

Measurements of schlieren patterns, required for the above determinations, were made using a Nikon Shadowgraph (model 6C) fitted with a projection screen and accurate to $2 \times 10^{-4}$ cm. Areas under peaks observed in sedimentation velocity were determined from these measurements by the method of trapezoidal integration and were corrected for radial dilution (Svedberg and Pedersen 1940). The total corrected area was related to the initial weight concentration of protein by assuming that the specific refractive increment of the protein was $1{\cdot}8 \times 10^{-3}$ dl/g (Perlmann and Longsworth 1948).

Methods for determining protein nitrogen, flocculation values, and levels of toxicity of the tetanus toxin followed standard procedures and have been described in detail previously (Dawson and Mauritzen 1967).

## III. Results

### (a) *Properties of Untreated Toxin*

The sedimentation velocity pattern obtained with tetanus toxin (0·95%) in 0·1M phosphate buffer (pH 7·0) at 20°C is shown in Figure 1. Essentially a single peak, characterized by $S_{20,w}$ of $6{\cdot}4_7$ S, is evident, together with a small amount of

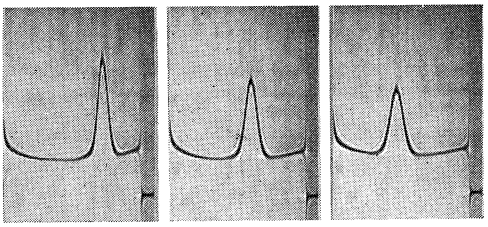

Fig. 1.—Sedimentation velocity patterns of tetanus toxin (0·95%) in phosphate buffer of ionic strength 0·22 and pH 7·0 at 20°C. The speed of rotation was 59,780 r.p.m., the bar angle was 60°, and the time interval between photographs was 16 min. Sedimentation is from right to left.

material sedimenting near the meniscus. In some preparations a small proportion (never greater than 5% of the total protein) of 10 S material was observed. The dependence of $S_{20,w}$ values of the major component on protein concentration (g/dl) is shown in Figure 2, the experimental conditions being those pertinent to Figure 1. The solid line in Figure 2 represents an attempt to fit the data by the method of least squares. On this basis, the data are described by the relation

$$S_{20,w} = 6{\cdot}7_3(1-0{\cdot}04c)\ \text{S} \qquad (1)$$

where the value of $6{\cdot}7_3$ S is the intercept on the ordinate axis of Figure 2 and represents the value extrapolated to infinite dilution, $S^0_{20,w}$, and $c$ (g/dl) is the total protein concentration.

The negative slope of the line shown in Figure 2 suggests that the system does not comprise a series of polymeric forms coexisting in rapid equilibrium (Schwert 1949; Nichol *et al.* 1964). Moreover, the concentration dependence is sufficiently small to permit analysis of the schlieren patterns of Figure 1 by the method of Fujita (1956) which applies to non-associating solutes exhibiting linear concentration dependence of small magnitude. The result is shown in Figure 3 in which the axes involve parameters discussed in detail by Baldwin (1957). It is clear from the height–area plot that the data from three exposures may be represented by a straight line passing close to the origin. Thus, it may be concluded that the material comprising the $6{\cdot}4_7$ S peak is homogeneous within the limits of the test. The slope of the line in Figure 3 is $2D/\omega^2S^0$, from which $D$, the apparent diffusion coefficient, was calculated as $4{\cdot}9\times10^{-7}$ cm$^2$/sec. It would be unwise to use this approximate value of

$D$ in a calculation of the molecular weight of the toxin. Indeed, comparison of molecular weights of aryl sulphatase A calculated on the basis of a Fujita analysis and determined accurately from sedimentation equilibrium data show that at least a 12% discrepancy may arise (Nichol and Roy 1964, 1965). Accordingly, the molecular weight of the tetanus toxin in 0·1M phosphate buffer, pH 7·0, was determined

Fig. 2

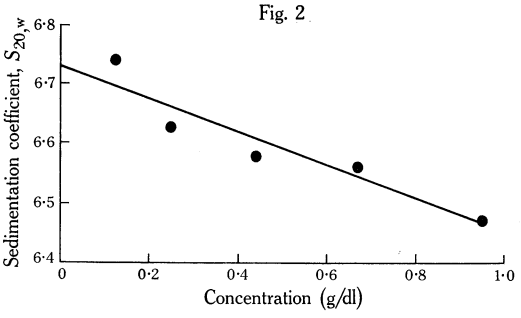


Fig. 3

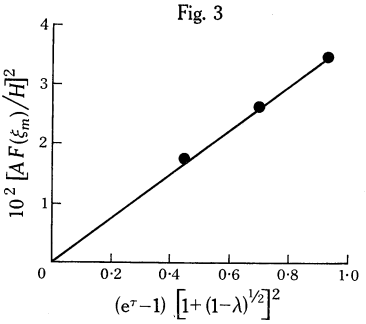


Fig. 2.—Variation of the sedimentation coefficient of tetanus toxin with concentration.

Fig. 3.—The height–area plot from which the diffusion coefficient, $D$, is evaluated. The slope of the line is $2D/\omega^2 S^0$. The symbols used on the axes are defined by Fujita (1956).

by the Archibald method. Values of $(dn/dx)_m/x_m c_m \omega^2$ obtained using 0·95 and 1·14% toxin solutions were independent of time, suggesting again that the material was essentially homogeneous with respect to molecular weight. The small amount of slow-sedimenting impurity (Fig. 1) would not be expected to be detected by this method. An average value of the molecular weight of 176,000±5,000 was obtained.

TABLE 1

EFFECT OF ENVIRONMENTAL PARAMETERS ON THE SEDIMENTATION PROPERTIES OF 1% SOLUTIONS OF TETANUS TOXIN

| Buffer | Ionic Strength | pH | Temperature (°C) | $S_{20,w}$ (S) |
|---|---|---|---|---|
| Phosphate | 0·22 | 7·0 | 4 | $6{\cdot}5_7$ |
| Phosphate | 0·22 | 7·0 | 20 | $6{\cdot}4_7$ |
| Phosphate | 0·05 | 7·0 | 20 | $7{\cdot}3_6$ |
| Acetate | 0·10 | 5·5 | 20 | $6{\cdot}7_0$ |
| Acetate | 0·05 | 5·5 | 20 | $7{\cdot}1_9$ |
| Diethylbarbiturate chloride | 0·10 | 8·2 | 20 | $6{\cdot}5_5$ |

The effects of varying temperature, ionic strength, and pH on the sedimentation properties of tetanus toxin (1·0%) were examined in selected environments and the results are summarized in Table 1. Several points require comment in relation to Table 1. First, the small difference in $S_{20,w}$ values observed at 4 and 20°C at pH 7·0 could be attributed to the large temperature correction (Svedberg and Pedersen 1940), required for comparison of the data. With other systems, lowering

the temperature has resulted in a marked decrease in $S_{20,w}$ values consistent with a weakening of intermolecular hydrophobic bonds (Kauzmann 1959; Nichol and Roy 1966). In this connection, it was also observed that introduction of sodium dodecyl sulphate (0·25% toxin and 0·2% detergent in 0·1M phosphate buffer, pH 7·0, at 20°C) failed to reveal any transformation of the ~ 7 S species to slower sedimenting forms. Secondly, there appears to be little pH-dependence of $S_{20,w}$ values in the pH range of 5·5–8·2. Thirdly, at pH values of both 7·0 and 5·5, a decrease in ionic strength resulted in an increase in $S_{20,w}$ values.

### (*b*) *Effect of Sulphite*

The sedimentation velocity pattern, shown in the upper section of Figure 4, was obtained with a 1·0% solution of tetanus toxin, which had been dialysed for 7 days at 0°C against buffer, pH 7·2, containing 0·03M sodium sulphite, 0·048M disodium hydrogen phosphate, and 0·02M sodium dihydrogen phosphate. Comparison with Figure 1, referring to similar pH and ionic strength values, shows that the pattern obtained in the presence of sulphite ions exhibits a marked asymmetry on the trailing edge. This is also reflected in the value of 5·90 S found for the weight-average sedimentation coefficient, $\bar{S}_{20,w}$, which is lower than the value calculated from the rate of movement of the maximum ordinate. The latter value compares favourably with those reported in Figure 2 and Table 1. Values of $\bar{S}_{20,w}$ obtained when 0·5 and 1·8% solutions of toxin were similarly treated were 5·60 and 5·80 S respectively. Thus the decrease in $\bar{S}_{20,w}$ observed was confirmed, and it was shown that there was no systematic variation in $\bar{S}_{20,w}$ with protein concentration. Graphical resolution of the patterns indicated that at each concentration approximately 20% of the material sedimented with an $S_{20,w}$ value in the vicinity of 4 S. The weight-average molecular weights of sulphite-treated toxin in the same environment were evaluated by the Archibald procedure. Values obtained using different protein concentrations, shown in parentheses, were 158,000 (0·4%), 156,000 (0·7%), and 152,000 (1·0%). In each case, values of $(dn/dx)_m/x_m c_m \omega^2$ decreased with time as is expected for a heterogeneous sample, and the reported values are those extrapolated to zero time. In separate studies at pH 7 with aged toxin samples containing ~ 5% 10 S material as well as the major component, it was found that addition of sulphite effected the complete removal of the 10 S material.

Sedimentation velocity analysis of a sample dialysed for 7 days against buffer containing 0·03M sodium sulphite and 0·1M acetate, pH 5·5, gave the result shown in the lower section of Figure 4. The isoelectric point of tetanus toxin is approximately pH 5·1 (Pillemer *et al.* 1948) and thus the net negative charge borne by the protein is less at pH 5·5 than at 7·2, and yet Figure 4 clearly shows that the sulphite has a greater effect at the higher pH value. The effect of sulphite ions was also observed at pH values of 6·1 and 9·0, but again was less pronounced than in the medium of pH 7·2 and of the same ionic strength, 0·25.

A comparison of flocculation and toxicity values was made between tetanus toxin stored for 7 days in phosphate buffer at pH 7·2 in the presence and absence of sulphite ions, the ionic strength being identical for each sample. It was found that the flocculation value of 2700 $L_f$ units per milligram of protein nitrogen remained

unchanged on sulphite treatment, but that at least 99% of the toxicity had been lost. In this connection, it was found that the addition to the toxin of $0{\cdot}25\times10^{-3}$M *p*-chloromercuribenzoate at pH 7, or of $0{\cdot}66\times10^{-3}$M iodoacetate at pH 8, had no significant effect on either the flocculation value or the level of toxicity. These reagents were also without effect on the sedimentation properties of the native toxin.

Fig. 4 Fig. 5

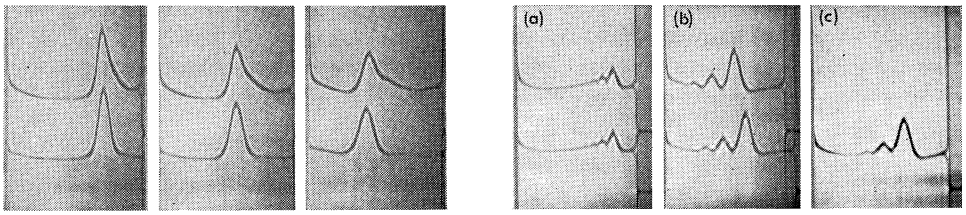


Fig. 4.—Sedimentation velocity patterns of tetanus toxin (1%) in phosphate–sulphite buffer, pH 7·2 (upper patterns), and in acetate–sulphite buffer, pH 5·5 (lower patterns). The speed of rotation was 59,780 r.p.m., the bar angle was 60°, and the time interval between photographs was 16 min. Sedimentation is from right to left.

Fig. 5.—Sedimentation velocity patterns of tetanus toxin at 20°C after modification with aldehydes: (*a*) 2% protein treated with 0·02M formaldehyde; (*b*) 2% protein treated with 0·02M glyoxal; (*c*) 1·5% protein treated with 0·002M glutaraldehyde. All experiments were performed in the presence of 0·1M phosphate, pH 7, the upper patterns referring to experiments in which the unreacted aldehyde was removed by dialysis, the lower ones [including (*c*)] to experiments with modifier present. In each case the bar angle was 60° and sedimentation is from right to left.

### (*c*) *Effect of Aldehydes*

Sedimentation velocity studies were performed on tetanus toxin subjected to treatment with the aldehydes formaldehyde, glyoxal, and glutaraldehyde. Typical results are shown in Figure 5. In relation to Figure 5(*a*), a 2% solution of toxin in 0·1M phosphate buffer, pH 7·0, was dialysed for 4 days at 37°C against the buffer containing 0·02M formaldehyde. A sample of the solution was dialysed against the phosphate buffer to remove any unreacted formaldehyde and subjected to sedimentation velocity analysis. The result is shown in the upper section of Figure 5(*a*). An analysis of the solution not dialysed to remove excess formaldehyde was performed simultaneously in a separate cell and the result is shown in the lower section of Figure 5(*a*). It is apparent that the aggregation of the 6·4 S material [evident as the major peak in Figure 5(*a*)] has been induced by the treatment. Indeed, there was considerable depletion of material recorded optically because of the sedimentation of large aggregates to the bottom of the cell in the early stages of the experiment. A control experiment in which the toxin was held at 37°C for 4 days without the addition of aldehyde revealed 95% $\sim$ 6 S material and only 5% of aggregate characterized by an $S_{20,w}$ of 10 S. Evidently, formaldehyde induces extensive and irreversible aggregation of the protein.

Experiments performed under identical conditions with 0·02M glyoxal yielded the patterns shown in Figure 5(*b*). It is evident that the extent of aggregation is less than that apparent in Figure 5(*a*); in particular, discrete sedimenting boundaries

with $S_{20,w}$ values of 6·4, 10, and 13 S are evident in Figure 5(*b*) and less depletion of the material is observed. In other experiments employing 0·05% toxin solutions in the same phosphate buffer and 0·01M formaldehyde or glyoxal, it was found that the material had been completely detoxified and aggregated to a lesser extent than shown in Figure 5 (only $\sim 10\%$ of 10 S material was apparent in the patterns together with $\sim 90\%$ of a 6·2 S species).

Finally, the result of treating the toxin with glutaraldehyde, shown in Figure 5(*c*), requires comment. A 1·5% solution of toxin in 0·1M phosphate buffer, pH 7·0, was made 0·002M with respect to glutaraldehyde by direct addition of the aldehyde, and held at 20°C for 3 hr prior to obtaining the sedimentation result. Again resolution of discrete peaks with $S_{20,w}$ values similar to those found in Figure 5(*b*) was observed. However, analysis of the solution after 5 hr of standing at 20°C revealed that the extent of aggregation had markedly increased. Under conditions employed for formaldehyde and glyoxal modification (37°C and 0·01 or 0·02M aldehyde) treatment with glutaraldehyde resulted in gel formation within 30 min. Even under the milder conditions relevant in Figure 5(*c*) no meaningful comparison of dialysed and undialysed samples could be made.

## IV. Discussion

The data summarized in Figure 3 and the invariance with time of the ratio $(dn/dx)_m/x_m c_m \omega^2$ found in Archibald experiments show that tetanus toxin, prepared by the method of Dawson and Mauritzen (1967), is essentially homogeneous with respect to molecular weight. A value of 176,000±5,000 for the molecular weight was obtained. This result, combined with the value $S_{20,w} = 6{\cdot}7$ S (Fig. 2), gives an estimated value of 1·5 for the frictional ratio, showing that the molecule is relatively asymmetric. The dependence of $S_{20,w}$ values on ionic strength, shown in Table 1, suggests that electrostatic forces may contribute significantly in determining the conformation of the unit with molecular weight of 176,000; it may also account for the scatter of $S_{20,w}$ values found by Raynaud, Turpin, and Bizzini (1960) from investigations in media of different ionic strengths. Bizzini *et al.* (1963) computed the disulphide and sulphydryl content of tetanus toxin by arbitrarily selecting a molecular weight of 100,000. Recalculation of their data indicates the presence of 3–4 disulphide bonds and 11 sulphydryl groups per mole of protein.

Since sulphite ions are specific for the scission of disulphide bonds (Cecil and McPhee 1959; Cecil and Loening 1960), a direct explanation of the results shown in Figure 4 is that sulphite cleaves a portion of bonds linking subunits of toxin in the unit with molecular weight 176,000. The interpretation is consistent with the molecular weight evaluations found at pH 7·2 in the presence and absence of sulphite ions. First, the observation that the *absolute* area of the slow sedimenting peak in the upper section of Figure 4 varied with total protein concentration excludes the possibility that the whole boundary is a bimodal reaction boundary, representing gradients of refractive index of species coexisting in rapid equilibrium (Nichol *et al.* 1964). Accordingly, the boundary is amenable to graphical resolution, which showed that the slower sedimenting material comprised $\sim 20\%$ of the total protein. Secondly, the calculated weight-average molecular weight of a system comprised of 80% and 20% of units with molecular weight 176,000 and 88,000 respectively is 158,000.

The model, which relates the slower sedimenting species to half-subunits of molecular weight 88,000, is consistent, therefore, with the weight-average values of 158,000–152,000 observed in the presence of sulphite. In addition, the observed $\bar{S}_{20,w}$ values are consistent with the model when the $S_{20,w}$ value of the subunit is taken as 4 S. The data are of insufficient accuracy to determine whether the postulated half-subunits are in fact identical with respect to molecular weight. However, they do exclude an alternative model that cleavage of disulphide bonds results exclusively in a shape rather than a size change.

It is clear that the cleavage of the disulphide bonds linking subunits of tetanus toxin is incomplete even at pH 7·2, where the maximal value of 20% slower sedimenting form was observed. An incomplete cleavage of disulphide bonds by sulphite in particular experimental environments has been reported for several other proteins (Cecil 1963). The approach and attack by sulphite ions is partly governed by the net charge in the vicinity of the disulphide bond (Cecil and Loening 1960; Bailey and Cole 1959); but studies with tetanus toxin at pH 5·5, a value close to the isoelectric point, failed to reveal increased relative amounts of subunit (lower section of Fig. 4). In this connection, it could be noted that the proportion of bisulphite ions to sulphite ions increases as the pH is lowered, and bisulphite ions are known to be less efficient cleaving agents (Cecil and McPhee 1959). The important point emerges, however, that treatment of the toxin at pH 7·2 with sulphite results in almost complete loss of toxicity, while 80% of the material retains the sedimentation properties characteristic of native toxin; clearly, the partial dissociation of the toxin is not the sole cause of the loss of toxicity. It is relevant to this observation that the cleavage of disulphide bonds by sulphite ions is heterolytic and produces sulphenyl sulphite ($-SSO_3^-$) and sulphydryl groups (Cecil and McPhee 1959). In this study, treatment with sulphite was prolonged for 7 days and no attempt was made to exclude atmospheric oxygen or metal ions. The conditions were deliberately selected to permit operation of the cyclic mechanism proposed by Bailey and Cole (1959) and Nichol and Creeth (1963) in which conversion of disulphide bonds, readily accessible to sulphite, to $-SSO_3^-$ groups is achieved. One (or more) of the four disulphide bonds in a molecule of toxin may differ from those linking the subunits in that it is readily accessible to attack with sulphite and according to the cyclic mechanism would be converted to the $-SSO_3^-$ form. This modification could affect the toxicity if the accessible disulphide groups were *either* directly involved in the toxic site *or* were structurally placed so that their modification to a charged form would lead to a conformational change of the protein, perturbating on the arrangement of the toxic site. The retention of flocculation value in the presence of sulphite is in accord with the generally accepted hypothesis that the antigenic properties of toxin are not exclusively governed by the groups involved in the toxic site.

The possibility that sulphydryl groups were directly involved in the toxic site was also examined. There is evidence that at least one of the eleven sulphydryl groups in a molecule of toxin is readily accessible. Thus, in aged toxin samples, 10 S material, corresponding to a dimer of 7 S species, is formed by intermolecular disulphide cross-linkage; the nature of the linkage is indicated by the removal of the 10 S material by the addition of sulphite (cf. Nichol and Creeth 1963). However, the addition of iodoacetate or *p*-chloromercuribenzoate resulted in no loss in toxicity.

The inference that accessible sulphydryl groups are not directly involved in the toxic site is in agreement with the findings of Bizzini *et al.* (1963).

The previously unreported aggregation of tetanus toxin induced by the additions of formaldehyde, glyoxal, and glutaraldehyde (Fig. 4) possibly proceeds by formation of methylenic linkages between amino groups and aromatic residues (Fraenkel-Conrat and Olcott 1948). The phenomenon appears to bear no direct relation to the detoxification of the protein, effected by the aldehydes, and a mechanism for their action in this respect awaits elucidation of the groups and their arrangement in the toxic site. It is hoped that the findings in this work that disulphide bonds (but not sulphydryl groups) are implicated at the toxic site or in maintaining the structural integrity of the site, and that the conformation of the unit with molecular weight 176,000 is influenced by interactions between charged groups (including possibly those introduced by sulphite treatment), may contribute to an understanding of the nature of the toxic site.

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