XLII. SULPHYDRYL GROUPS IN PROTEINS

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THE work recorded here is an attempt to obtain quantitative information concerning the sulphydryl groups of proteins. It has long been realized that the combination in which sulphur exists in cystine and methionine is incapable of explaining all the reactions of protein sulphur. As instances of such reactions may be mentioned the suspected existence of free sulphydryl groups in protein (cysteine never having been isolated from protein), the recognition of labile and non-labile sulphur, recently the subject of yet further researches [Blumenthal & Clarke, 1935] and the exposure of sulphydryl groups on denaturation of protein. Certain alternative suggestions have been put forward; e.g. the —CS—NH— linkage of Harris [1923] and the —CO—S— linkage of Mastin & Schryver [1926]; but direct experimental support of such hypotheses is lacking. On the contrary, modern work [e.g. Speakman & Whewell, 1936] seems to favour simple hydrolysis of the disulphide linkage as the explanation of the sulphydryl group on denaturation.

We ourselves were more particularly interested in the distribution of sulphur in muscle proteins. That muscular tissue gives a nitroprusside reaction is an old-established fact and that muscle powder (i.e. denatured protein) contains a thermostable sulphydryl group was recorded by Dixon & Hopkins [1922]; whilst more recently Astbury [1934] has commented upon the similarity of the X-ray diffraction pictures of keratin (which is rich in cystine) and the muscle protein myosin, without, however, attributing functional significance to the sulphur groupings. The reactivity of the sulphydryl group and the simultaneous presence of glutathione in close association with the muscle proteins offer free play to the imagination. There seemed, however, little hope of eliciting useful information from muscle unless some quantitative method of estimating sulphydryl could be applied. The present communication is concerned with such a method and the preliminary results of its application. Whilst this work was in progress Mirsky & Anson [1935] published an account of their work directed to the same end but using quite different methods. A preliminary account of our own work has already appeared [Todrick & Walker, 1936].

Method

Briefly the method consists in measuring the amount of oxidation-reduction indicator, phenolindo-2:6-dichlorophenol, reduced by a known weight of protein. A series of tubes was set up containing the same known amount of protein, of the order of 80 mg. Varying amounts of indicator solution (0.1–1.0 ml.) were added and, in the case of denatured proteins, the tubes were then immersed in a water-bath at 100° for a specified time (20 min.). The tubes whose contents were decolorized within that time were noted and a further series of tubes was then set up containing a closer range of quantities of indicator. In this way it

was possible to ascertain the precise quantity of oxidation-reduction indicator required to bring about the oxidation of the sulphhydryl group. The arbitrary period of 20 min. was selected as the result of plotting time against amount of indicator, when it became clear that decoloration was complete in about 10 min. at 100°; 20 min. were therefore allowed in the standard procedure to make sure that oxidation of sulphhydryl was complete and within this time no reoxidation takes place. In the experiments with myosin, which had to be carried out at 38°, the reaction was much slower and consequently a longer period (12 hours) was necessary to ensure complete oxidation of sulphhydryl. With this prolonged period it was necessary to guard against reoxidation of the indicator; the tests were therefore carried out in an inert atmosphere.

The indicator was standardized in terms of cysteine. Cysteine hydrochloride was twice recrystallized from pure hydrochloric acid (Kahlbaum's) and dried *in vacuo* over solid NaOH. A weighed amount of the pure hydrochloride was first neutralized, in an atmosphere of nitrogen, and then titrated, also in nitrogen, with a solution of phenolindo-2:6-dichlorophenol, approximately 0.1%. Towards the end the titration was carried out with the receiving vessel immersed in a bath of boiling water, in order to expedite the reaction and also to simulate the reaction with proteins. At the beginning of these experiments our solutions showed that 1 mg. of cysteine was equivalent to 2.85 mg. of indicator; whilst at the end, using different solutions from the originals, we found the equivalent to be 2.82 mg. All the results quoted were reproducible, but taking everything into consideration, including the difficult end-point, it is probable that the estimations are only accurate within 2-3%. The results themselves afford justification for the conclusion that the measurements made, under the conditions stated, are specific for sulphhydryl groups.

Results

Ovalbumin. This was prepared in the usual way from the whites of fresh eggs by half-saturation with ammonium sulphate and precipitation of ovalbumin from the filtrate by full saturation. The latter precipitate was redissolved, filtered and reprecipitated. The final precipitate was then dialysed against distilled water until the dialysate no longer gave any test for sulphate. The concentration of protein was determined by the nitrogen content (micro-Kjeldahl) allowing for a small amount of nitrogen due to residual traces of ammonium sulphate and assuming 15.4% N for ovalbumin. Three solutions used were found to contain 39.9, 45.45 and 54.65 mg. ovalbumin per ml. respectively. The pH of the solutions was 6-7. It is not proposed to give the detail of the estimations in each case, as this consists mostly of slight variations in the amounts of protein and indicator used. In general 1-2 ml. of the above protein solutions were used, requiring 0.5-1 ml. of indicator solution. Four typical results for denatured ovalbumin gave the following values for the cysteine content: 0.597, 0.676, 0.622 and 0.62%. Mean value, 0.63%. In all the above cases denaturation was effected by immersion of the tubes in the water-bath at 100°. One estimation was carried out at 40° and revealed, as was to be expected, no apparent cysteine.

Serum albumin. This was prepared by the same general methods as ovalbumin. The solution finally used contained 24.2 mg. of protein per ml., based on a nitrogen content of serum albumin of 15.15%. This protein on denaturation by heat had no detectable effect on the indicator. This is in conformity with the fact that denatured serum albumin gives no sulphhydryl reaction; although, as pointed out by Walker [1925] it does now give a disulphide reaction, which it did not give in the native state. This experiment, despite the negative result, is

considered to be important as it establishes that the indicator is not affected by any groups in a typical denatured protein, other than cysteine. It was noted that the coagulum of denatured serum albumin was sufficiently acid to change the indicator, in the adsorbed state, from blue to red, although the medium in which coagulation took place was still at *pH* 6–7. It is possible that this effect is due to the sulphenic acid groups appearing in coagulation.

Myosin. There has been considerable confusion in the past in connexion with the muscle proteins because different workers have often been dealing with different preparations under the name of myosin. In order to facilitate the adoption of a standard method we have employed that described by Edsall [1930], which has also been used by Smith [1933]. Briefly, it consists in killing a rabbit and immediately perfusing the carcass with saline. The back and leg muscles are then quickly removed, minced and extracted with 1.2*M* KCl, buffered with phosphate at *pH* 7.5. The soluble protein is precipitated by dilution of the filtered extract with 20 volumes of distilled water; the precipitate is centrifuged, redissolved in the extraction mixture, filtered and reprecipitated by dilution. This precipitation process is repeated three times. The final precipitate is dissolved in the buffered salt solution and filtered and is then ready for use. All solutions and apparatus used must be cooled beforehand. Any deviations from the method may lead to different products being obtained; even room temperature is apt to lead to the formation of a certain amount of insoluble material. Our preparations of myosin were always kept *in vacuo* in cold store when not in use and were always centrifuged immediately before use. In our earlier experiments the concentration of protein was determined by drying and weighing an aliquot portion of the mixture. This was because no reliable analytical figures were available for myosin. Later we prepared a specimen of myosin in as pure state as possible and had it analysed, with the following results: N, 15.3, S, 0.94, ash 0.3%. The high ash content is attributable to the fact that myosin is only soluble in salt solution and forms a very gelatinous precipitate on dialysis, from which it is difficult to remove the last traces of salt. The N and S values are calculated on an ash-free basis. We made use of the above figure for N in calculating the protein concentration in all the figures quoted in this account.

The solution of myosin thus prepared was found to give a faintly positive nitroprusside reaction. That this could not be due to glutathione is indicated by the following facts:

- (1) The suspended carcass of the rabbit was always thoroughly perfused, saline entering the aorta and leaving by the right auricle.
- (2) The mode of preparation of the myosin involved precipitation of the protein by dilution with 20 volumes of water, three times.
- (3) No glutathione could be detected in the dialysate from a specimen of myosin.

We therefore proceeded to estimate the sulphydryl content of the myosin, not quite in the same manner as for the other proteins, for the following reasons. First, there was the obvious lability of the sulphydryl group, as will be seen presently, and secondly, the estimations being on the native protein, the temperature had to be kept below 40°. This meant that the reaction was much slower than in the case of denatured proteins at 100°. Measurements on myosin were therefore carried out in Thunberg tubes, which were thrice evacuated and filled with nitrogen after all the ingredients had been added. The tubes were left for 12 hours before making a final decision on the colour. Our two earliest estimations gave values of 0.17 and 0.14% cysteine, on two different rabbits, and we soon formed the impression that, despite working as rapidly as possible and at

a low temperature, the preparation was undergoing oxidation of the initially free sulphydryl. It should perhaps be mentioned that, taking the bulk of the muscle from one rabbit and working it all up, the final myosin was not obtained until 5 or 6 days from the start. We therefore modified the procedure whereby, taking only a small amount of protein, the complete process could be accomplished in a few hours. Our subsequent results, each on different rabbits, were: 0.18, 0.24, 0.24, 0.27 % cysteine. That oxidation does occur we have confirmed by redetermination of a preparation which initially gave 0.27 % and after a few days 0.20 %; another, unpurified, gave 0.24 %, falling in 3 days to 0.11 %.

So far, we have only made approximate measurements of myosin after heat-denaturation and these gave results varying but little from the native protein. We have done one experiment on the myosin of a rabbit killed by injection of sodium iodoacetate; this revealed no significant variation (0.23 %) from the normal.

DISCUSSION

The results for ovalbumin and serum albumin are primarily of interest in establishing the method. Neither protein in the native state affects the indicator; nor does denatured serum albumin (containing all the typical amino-acids, including cystine) affect it; whilst denatured ovalbumin is quickly oxidized by it. The only significant difference between the two denatured proteins is the presence of the sulphydryl group in the latter.

The total cystine (including any cysteine) in ovalbumin is given as 2.27 % by Baernstein [1932]; our finding of 0.63 % cysteine would thus account for some 28 % of it. There are no figures for cysteine in the literature except those of Mirsky & Anson [1935] and it seems to us that the methods employed by these workers are open to objection, notably on account of the chances of oxidation having occurred. Thus in their methods, there is much washing and centrifuging and mechanical stirring of the protein after denaturation. In their "direct" method the protein is exposed to a solution of cystine more alkaline than pH 9.0. In their "indirect" method the protein is also precipitated with acid acetone, ground in a hot mortar and finally heated at 108° for 30 min.; it is then hydrolysed for 15 hours with 5*N* H₂SO₄ without any stated precautions against oxidation. The "indirect method" gives a higher value (0.616 %) for the cysteine content of denatured ovalbumin than the "direct" method (0.55 %). In both methods the cysteine is estimated colorimetrically.

When we come to consider myosin, the chief interest is the existence of free sulphydryl in the native protein. The amount of sulphydryl is small; our highest value, 0.27 % cysteine,¹ corresponds only to some 13–17 % of the total cystine, using Baernstein's figure for the cystine content of halibut muscle for the calculation; but the result is definite and, to us, significant. First, there is here demonstrated a free sulphydryl group in a native protein; and secondly, the existence of such a group is in conformity with the lability which might be expected from a protein exhibiting the various physical and physiological changes of muscle. The explanation of the intimate nature of contraction must reside in the properties of the proteins of muscle; and in myosin we have a protein which seems to embody all the physical properties of muscle. Although it is less in quantity than myogen (1 : 3), there is much evidence to show that it

¹ Mirsky [1936], using the methods already referred to, has investigated muscle proteins. He makes no categorical statement concerning sulphydryl in myosin; but it may be calculated from one of his tables that native rabbit myosin contains some 0.3 % cysteine at the pH (7.6) at which we worked.

plays a more dominant role than the latter. Thus it is probable that the fibrils are largely composed of myosin and these occupy some 50 % of the volume of the muscle; whilst there is also the possibility that myosin occurs in thicknesses of the order of surface films [Smith, 1933]. Boehm & Weber [1932] hold myosin mainly responsible for the diffraction pattern of muscles, whilst von Muralt [1932] concludes that myosin occupies the *Q*-bands of the fibril, myogen being confined to the sarcoplasm.

SUMMARY

1. A method for the estimation of the sulphydryl content of proteins is described.

2. Native serum albumin and ovalbumin and denatured serum albumin contain no free sulphydryl. Denatured ovalbumin contains free sulphydryl to an amount corresponding with 0.63 % cysteine.

3. Native myosin contains free sulphydryl to the extent of 0.27 %. The significance of this finding is discussed.

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