THE REACTION BETWEEN IODOACETIC ACID AND
DENATURED EGG ALBUMIN *

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(Received for publication, October 23, 1939)

The reaction between iodoacetic acid and sulfhydryl compounds
has received much attention in recent years. Dickens (1) has
shown that this reaction proceeds with the formation of HI and
the substitution of a carboxymethyl group for the hydrogen of
the thiol group. Smythe (2) has measured the rate of reaction of
iodoacetic acid with various sulfhydryl compounds by estimation
of the carbon dioxide change in a CO₂-bicarbonate buffer as a
result of the HI produced. Rapkine (3) showed that iodoacetic
acid may react not only with the sulfhydryl groups of relatively
simple molecules, such as cysteine and glutathione, but also with
those of proteins. It was found by Rapkine that as denatured
egg albumin stood with increasing amounts of iodoacetic acid the
intensity of its nitroprusside test diminished until it finally dis-
appeared. Mirsky and Anson (4) have used this reaction in their
method for determining available thiol groups in proteins. These
investigators found that iodoacetic acid might react with only a
portion or with none of the sulfhydryl groups of native proteins,
as evidenced by the continued presence after the reaction of part
or all of the original protein cysteine in the protein hydrolysate.
Denaturation of the protein, however, causes all of the sulfhydryl
groups to be capable of interaction with iodoacetic acid, there
being, in this case, no free cysteine in the protein hydrolysate
after the reaction.

In the experiments of Mirsky and Anson iodoacetic acid was
allowed to react with coagulated denatured egg albumin; these

* This research was aided by a grant from the American Academy of
Arts and Sciences.
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authors state that 3 hours are required for the reaction to go to completion. In the present work, however, the denatured egg albumin was not permitted to coagulate. This was accomplished by adding 0.01 N KOH to a pure solution of egg albumin (prepared from the whites of fresh eggs by the method of Kekwick and Canaan (5)) to pH 7.3. The mixture was then placed for 10 minutes in water which had been brought to a boil. That iodoacetic acid reacts with this solution of denatured egg albumin much more rapidly than with a coagulum will be shown later. It is obvious that the interaction between iodoacetic acid and protein may be followed by measuring the iodide produced by the reaction. To this end a simple method for determination of iodides was devised.

The experiments were carried out in the following manner. To 3.5 cc. of egg albumin solution containing a known amount of the protein were added 1.5 cc. of 1 M phosphate buffer, pH 7.3, and 5 cc. of approximately 0.1 N iodoacetic acid which was previously neutralized with KOH. The reaction between the iodoacetate and protein was permitted to proceed for the desired period of time, at the end of which 0.25 cc. of concentrated H$_2$SO$_4$ and 0.25 cc. of 100 per cent trichloroacetic acid (10 gm. of trichloroacetic acid dissolved in water to make 10 cc. of solution) were added. The mixture was filtered and 0.1 cc. of 3 per cent H$_2$O$_2$ added to the filtrate. The depth of color of the iodine solution produced was then estimated, at the point of its maximum development, in a photometer, and the amount of iodide read from a previously prepared standardization curve. A blank was run with each experiment, which cancelled any iodide that was released by the iodoacetate itself.¹

A typical experiment in which periods of reaction time from 1 minute to 6 hours were studied is shown in Fig. 1, Curve A. It may be seen that there is an initial rapid production of iodide lasting no longer than 10 minutes, after which the reaction slackens and after 40 minutes proceeds at a steady, much slower pace.

¹ A Sheard and Sanford photometer (Central Scientific Company) was used in these experiments. By this method quantities as low as 0.05 mg. of iodide in 10.5 cc. of solution could be estimated. In the region of 0.2 to 0.5 mg. of iodide, where most of the determinations fell, known amounts of iodide could be checked within 0.007 mg.
The initial sharp rise in iodide is without doubt mainly a result of the interaction between the iodoacetate and the thiol groups of the protein. The cause of the continued slower rate of iodide production is, however, still a matter of conjecture. The work of Michaelis and Schubert (6) suggests the possibility that it results from the action of iodoacetate on amino groups of the denatured egg albumin. However, cystine, tyrosine, arginine, and glutamic acid when subjected to the same experimental procedure yielded iodide at a rate much slower than would be required by this explanation. This observation, on the other hand, does not rule out the possibility of greater reactivity of the amino groups in the protein molecule under these conditions.

\[ \text{Mg. I}^- \times \frac{\text{mol. wt. cysteine}}{\text{mol. wt. I}} = \frac{0.95}{\text{mg. egg albumin}} = \% \text{ cysteine} \]

Curve A, heat-denatured egg albumin which was treated with iodoacetate immediately following denaturation; Curve B, heat-denatured egg albumin which was treated with iodoacetate after standing 4 hours following denaturation; Curve C, urea-denatured egg albumin which was treated with iodoacetate after 1 hour in urea solution; Curve D, urea-denatured egg albumin which was treated with iodoacetate after 20 hours in urea solution.

\(^2\) The nitroprusside test was undiscernible after a reaction period of 5 minutes.
It is of interest to note that when a solution of native egg albumin was tested in the same manner as was the denatured protein no iodide was found at the end of a 5 hour period in the presence of iodoacetate. This indicates that not only the sulfhydryl but all of the groups in the denatured egg albumin which produce iodide with iodoacetate are non-reactive toward this substance in the native protein. Anson (7), however, has shown that they may not be inert toward all reagents.

It may also be observed from Fig. 1, Curve A, that groups other than sulfhydryl produce iodide as a straight line function with time. If this line for non-sulfhydryl iodide be extrapolated to zero time, a value for the iodide which results from reaction of the sulfhydryl groups is obtained. Assuming all of these sulfhydryl groups to be part of the cysteine moiety of the protein molecule, an index to the cysteine content of the protein is thus obtained. This treatment gives a value in heat-denatured egg albumin of 0.55 per cent available cysteine (average of ten determinations with a range of 0.53 to 0.57 per cent cysteine). This figure is in good agreement with those of Mirsky and Anson, 0.56 to 0.61 per cent (4), Todrick and Walker, 0.63 per cent (8), Kuhn and Desnuelle, 0.58 per cent (9), and Greenstein, 0.50 per cent (10).

The interesting observation of Greenstein (10) that denaturation of egg albumin by urea, guanidine, and various derivatives makes available a greater number of thiol groups than does heat denaturation made it appear worth while to study the reaction between iodoacetic acid and urea-denatured egg albumin. In these experiments 2 cc. of egg albumin solution (containing 30 to 90 mg. of egg albumin) were added to 2.4 gm. of urea, and the solution permitted to stand at 24–26°C for 1 hour. (Greenstein has shown that the maximum number of —SH groups appears within half an hour.) Phosphate buffer and iodoacetate were then added and the determinations carried out as previously described.

It may be noted from Fig. 1, Curve C, which shows data from a typical experiment, that the initial rapid rise in iodide (sulfhydryl) is markedly greater than that given by heat-denatured egg albumin. Extrapolation of non-sulfhydryl iodide to zero time yields a value of 0.87 per cent cysteine (average of eight determinations with a range of 0.82 to 0.91 per cent cysteine). This figure is lower than
that determined by Greenstein (about 1.00 per cent) by titration with porphyrindin dye. It is also of interest to note that the slope of the rate of production of non-sulfhydryl iodide is greater than that found with heat-denatured egg albumin. Thus, to the characteristics of urea denaturation as compared with heat denaturation may be added the observation that it causes a greater increase in availability not only of sulfhydryl groups but of other iodoacetate-reacting groups as well.

That the thiol groups of a solution of denatured egg albumin are quite labile is shown by the data presented in Fig. 1, Curves B and D. It was found that if heat-denatured egg albumin was permitted to stand for some hours (at pH 7.3) before being treated with iodoacetate the cysteine content calculated from the iodide released was less than that usually determined. That this disappearance of sulfhydryl groups does not represent a reversal of the denaturation of egg albumin (which has never been demonstrated) is evidenced by the fact that reheating of the solution, which would in this case redenature the egg albumin and give the original value for cysteine, caused no increase whatever in the sulfhydryl groups. This same phenomenon is also shown by urea-denatured egg albumin. On standing for 20 hours in urea the available sulfhydryl groups were markedly decreased. Since it is scarcely possible that denaturation could be reversed under these circumstances, this disappearance of the sulfhydryl groups probably indicates their oxidation.3 On the other hand, the non-sulfhydryl groups which yield iodide with iodoacetate are apparently not thus susceptible to destruction, since their rate of iodide production is not diminished after standing.

SUMMARY

1. The reaction between iodoacetic acid and denatured egg albumin was studied by measurement of the iodide produced by the reaction.

2. In the course of this reaction there is an initial rapid production of iodide, which is believed to be caused by the sulfhydryl groups, followed by a slower steady yield of iodide due to some

3 Hopkins (11) showed that the nitroprusside reaction of urea-denatured egg albumin eventually disappears on standing but that treatment with reducing agents causes it to return.
other group or groups as yet unidentified. Extrapolation of this non-sulfhydryl iodide to zero time yields a value for available cysteine of 0.55 per cent in heat-denatured and 0.87 per cent in urea-denatured egg albumin.

3. The sulfhydryl groups of the denatured egg albumin are labile, measurably diminishing in a few hours.

The author wishes to acknowledge with thanks the criticism and suggestions of Dr. H. B. Bull as well as the help of Dr. S. R. Gifford in the accomplishment of this work.

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