REATIONS OF XANTHYDROL

II. INSULIN, LYSOZYME, AND RIBONUCLEASE*

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Considerable information concerning the active centers of biologically active proteins has been obtained through the use of reagents which react with one or more of the amino acid side chains (R groups) of the protein (2, 3). With few exceptions, none of these reagents exhibit complete specificity; i.e., they react with but one kind of amino acid residue in the protein. Despite this disadvantage, isolation and analysis of a chemically modified protein can often yield valuable information about the protein and its essential R groups (4–6).

Xanthydrol falls in the group of non-specific reagents since it reacts with seven individual amino acids in acetic acid solution (7). With proteins, however, xanthydrol reacts with fewer R groups than are theoretically possible. The estimation of the number of xanthyl groups which are bound to a protein utilizes the finding that in acid solution xanthyl groups migrate to the indole nucleus of tryptophan to form the colored, acid-stable substance, xanthyltryptophan. This phenomenon is termed transxanthylatation.

Xanthylinsulin, xanthyllysozyme, and xanthylribonuclease have been isolated. Xanthylinsulin is almost as active as insulin in causing hypoglycemia in mice and rabbits, xanthylribonuclease exhibits but a small fraction of its original activity, and xanthyllysozyme is completely inactive in the lysis of Sarcina lutea.

EXPERIMENTAL

Materials—Crystalline lysozyme (Armour), crystalline ribonuclease (Armour), and crystalline zinc insulin (Armour, lot No. 437-27) were used in this work. Xanthydrol was synthesized from phenyl salicylate (8) or purchased. It was dissolved in glacial acetic acid and used on the day of preparation. Xanthylacetamide, m.p. 244–247°, was synthesized by the procedure of Phillips and Pitt (9). S-Xanthylecysteine hydrochloride was synthesized in this laboratory (7).

* This investigation was supported in part by research grants from the National Institutes of Health, United States Public Health Service, RG-1765, and from Armour and Company. A preliminary report of this work was presented at the meeting of the American Society of Biological Chemists, Cleveland, Ohio, 1951 (1).
Xanthylation of Proteins—The protein was weighed, placed in a centrifuge tube, and dissolved in a minimal volume of water. Xanthydrol (100 moles per mole of protein) was dissolved in glacial acetic acid and added to the protein solution. The final acetic acid concentration varied from 75 to 95 per cent. The mixture was maintained at 37° for 1 hour and anhydrous ether was then added to precipitate the xanthyl protein and to extract unreacted xanthydrol. The mixture was centrifuged, washed twice with ether, and the product dried in vacuo overnight over KOH and P₂O₅.

Determination of Total Xanthyl Groups of Xanthyl Proteins—0.5 ml. of dl-tryptophan in H₂O (1 mg. per ml.) was added to 0.5 ml. of the xanthyl protein solution (3 mg. per ml.) in a Pyrex test-tube calibrated at 5.0 ml. To this solution 2.5 ml. of concentrated HCl were added. The solution was diluted to 5.0 ml. with H₂O and mixed, a marble was placed on top of the tube, and it was heated in a boiling water bath for 1 hour. The solution was cooled, extracted twice with wet benzene, and the benzene evaporated from the aqueous phase by a stream of air. It was brought to volume with 6 N HCl, transferred to a 15 × 125 mm. cuvette, and the absorbancy at 510 mμ determined with a Coleman jr. spectrophotometer. The number of xanthyl groups per mole of protein was determined from the standard curve (Fig. 1). This curve is based on transxanthylation from xanthylacetamide and S-xanthylcysteine to tryptophan under the above conditions. The fact that these two dissimilar xanthyl derivatives form equimolar quantities of xanthyltryptophan under these conditions furnishes support for this method of xanthyl group determination. Transxanthylation studies with all of the xanthyl amino acids will be required, however, to demonstrate the general validity of this method.

Determination of Biological Activity of Xanthyl Proteins—Lysozyme activity was determined by the method of Dickman and Proctor (10). Ribonuclease activity was determined by a micromodification of the method of McCarty (11). Insulin activity was determined by the standard mouse assay.

Results

Xanthylation of Insulin—The rate of reaction of xanthydrol with insulin was followed by spectral-absorbancy measurements. As demonstrated in Fig. 2, an appreciable reaction had occurred in 1 minute, and it was essentially complete in 30 minutes. Xanthylinsulin was isolated and tested for hypoglycemic activity in both mouse and rabbit tests. The values of the mouse assays (Table I) indicate that xanthylinsulin is almost as active as the control sample. Xanthylinsulin was found to contain 2.2 xanthyl groups per mole in the transxanthylation procedure. In contrast to insulin, xanthylinsulin is relatively insoluble at pH 7.4. It did not, however,
show evidence of prolonged hypoglycemic activity in the rabbit. It is, of course, possible that the xanthyl residues have been removed \textit{in vivo}. To test the stability of xanthylinsulin at physiological pH and temperature, it was incubated in phosphate buffer, pH 7.4, for 2 hours at 38°, then extracted

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{Formation of xanthyltryptophan by transxanthylation. \textbullet, absorbancy derived from xanthylacetamide; \textcircled{O}, absorbancy derived from \textit{S}-xanthylcysteine.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig2.png}
\caption{Rate of reaction of insulin with xanthydrol. The reaction mixture contained 25 mg. of insulin and 50 mg. of xanthydrol in 3 ml. of 83 per cent acetic acid. At the time indicated, 0.5 ml. was treated with anhydrous ether and the precipitate washed five times with anhydrous ether at the centrifuge. The ether-insoluble residue was dissolved in 10 ml. of 10 per cent acetic acid and its spectrum measured with a Cary recording spectrophotometer. Curve 1, xanthydrol, $10^{-4}$ \textit{M}, in 60 per cent acetic acid; Curve 2, insulin, zero time control; Curve 3, insulin, 1 minute; Curve 4, insulin, 30 minutes; Curve 5, insulin, 18 hours.}
\end{figure}
three times with ether to remove any liberated xanthyl. The spectral-absorbancy curve of the aqueous phase indicated that less than 5 per cent of the xanthyl groups had been removed. These results, however, do not exclude the possibility that dexanthylation in vivo has occurred.

**Table I**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Insulin</th>
<th>Xanthylinsulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.3</td>
<td>14.6</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>21.2</td>
</tr>
</tbody>
</table>

Mouse assay in units per mg.; standard error, 15 per cent. Activity determined by Armour and Company.

**Fig. 3**

**Fig. 4**

**Fig. 3.** Inactivation of lysozyme by xanthyl. The reaction mixtures contained lysozyme, $3 \times 10^{-4} \text{M}$, and xanthyl as indicated, in 95 per cent acetic acid. The solution was maintained at $20^\circ$ for 60 minutes, then extracted three times with ether, and the aqueous phase neutralized and lysozyme activity determined on an aliquot.

**Fig. 4.** Rate of inactivation of lysozyme by xanthyl. The reaction mixtures contained lysozyme, $3 \times 10^{-4} \text{M}$ in 95 per cent acetic acid and xanthyl. Curve 1, $10^{-3} \text{M}$; Curve 2, $5 \times 10^{-3} \text{M}$; Curve 3, $10^{-2} \text{M}$. Temperature, $20^\circ$. The solutions were treated as stated in Fig. 3.

**Xanthylation of Lysozyme—**Various quantities of xanthyl were added to lysozyme ($3 \times 10^{-4} \text{M}$) in 95 per cent acetic acid. The reaction mixture was maintained for 60 minutes at $20^\circ$, at which time the xanthyllysozyme was precipitated by the addition of anhydrous ether and washed twice more with anhydrous ether. As shown in Fig. 3, 38 per cent inactivation was caused by $10^{-3} \text{M}$ xanthyl and 75 per cent by $5 \times 10^{-3} \text{M}$ concentra-
tion of the reagent. When the rate of lysozyme inactivation was determined at three different xanthydrol concentrations, the data of Fig. 4 were secured. With $10^{-3}$ M xanthydrol, only a small increase in lysozyme inactivation occurred between 30 and 60 minutes, but with $5 \times 10^{-3}$ M xanthydrol the rate of inactivation was almost linear. It is apparent that the concentration of xanthydrol in the reaction mixture markedly influenced both the rate and the extent of the reaction. A sample of inactive xanthyllysozyme was assayed for xanthyl groups by the transxanthylation procedure. A value of nine xanthyl groups per mole was obtained. The absorption spectra of lysozyme and of isolated xanthyllysozyme are presented in Fig. 5.

![Ultraviolet spectral-absorbancy curves.](http://www.jbc.org/)

**Fig. 5.** Ultraviolet spectral-absorbancy curves. Curve 1, xanthylribonuclease, 0.63 mg. per ml.; Curve 2, ribonuclease, 1.23 mg. per ml.; Curve 3, xanthyllysozyme, 0.3 mg. per ml.; Curve 4, lysozyme, 0.3 mg. per ml. Solvent, 0.1 N HCl.

**Xanthylation of Ribonuclease**—Ribonuclease was treated with a 100 times molar excess of xanthydrol for 1 hour in acetic acid solution. The product was precipitated with ether and the ultraviolet spectral-absorbancy curve compared to that of the untreated enzyme (Fig. 5). The customary shift in the peak towards the longer wave-lengths is evident, as well as an increase in the absorptivity. This material contained 5.5 xanthyl groups per mole and possessed 2.4 per cent of the activity of the control (Table II). Other xanthylribonuclease were prepared with lower concentrations of xanthydrol in the reaction mixtures. Fewer xanthyl residues were incorporated on the protein, and higher ribonuclease activities were found with these samples.

Most of the xanthyl amino acids are unstable in hot, dilute acid solution (12) but ribonuclease is unusually stable under these conditions (13, 14). A xanthylribonuclease solution (5.5 xanthyl groups per mole) was adjusted to pH 3 and aliquots were placed in a boiling water bath for 5 and 15
REACTIONS OF XANTHYDROL. 11

minutes. Each was cooled, extracted with ether, and the ribonuclease activity determined. The lower portion of Table II indicates that a significant reactivation was accomplished by this treatment.

**Table II**

*Effect of Xanthylation and Dexanthylation on Ribonuclease Activity*

The three xanthylribonucleases were prepared by treating ribonuclease with 100, 25, and 10 times molar excess of xanthyl in glacial acetic acid for 1 hour at 37°. Each was precipitated by ether, washed two times with ether, and dried in vacuo. The control was treated in the same fashion but in the absence of xanthyl.

<table>
<thead>
<tr>
<th>Xanthyl groups per mole</th>
<th>Ribonuclease activity</th>
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<tr>
<td></td>
<td>Activity expressed as per cent control ribonuclease</td>
</tr>
<tr>
<td></td>
<td>per cent</td>
</tr>
<tr>
<td>0 (control)</td>
<td>100</td>
</tr>
<tr>
<td>5.5</td>
<td>2.4</td>
</tr>
<tr>
<td>3.5</td>
<td>4.2</td>
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<tr>
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<td>12.7</td>
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**DISCUSSION**

The wide range of effects obtained with three biologically active proteins, in conjunction with the results secured with cytochrome c (15), suggests that xanthyl may become a useful reagent in the investigation of protein structure as related to biological activity. In a partially reversible inactivation such as was found with ribonuclease, the determination of the number and location of the xanthyl groups may yield some insight into the active R groups of the enzyme.

Unfortunately, the solubility properties of xanthyl limit its use to proteins which are soluble and are not inactivated in concentrated acetic acid solutions. This limitation can probably be overcome by the introduction of polar groups on the aromatic rings of the reagent and thus can increase its solubility in water.

Both xanthyl and xanthyde and numerous derivatives are being intensively investigated as chemotherapeutic agents, insecticides, pesticides, etc. (16–21). Miracil C, for example, has the structure 1-diethylaminoethylamino-4-methylxanthyl, and the even more effective Miracil D is
a thiaxanthone derivative (17). Although the mechanism of action of such agents is unknown, it is possible that the antibiological effects of these compounds are due to their inactivation or inhibition of essential enzymes. The question whether xanthones exert their effects directly or after reduction in vivo to xanthydrols also cannot be answered at present. The ability of xanthydrol to react directly with proteins favors the latter alternative.

We wish to thank Dr. E. E. Hays and Armour and Company for generous gifts of lysozyme, insulin, and ribonuclease, and for carrying out the insulin assays.

SUMMARY

The reaction of xanthydrol with the proteins insulin, lysozyme, and ribonuclease has been studied. Evidence that a reaction takes place includes spectrophotometric data, determination of xanthyl groups bound to protein, and measurement of the biological activity of the xanthyl proteins. Lysozyme was completely inactivated by treatment with xanthydrol, ribonuclease was decreased to 2 per cent of its original activity, and insulin, as tested in vivo, was but slightly affected. The relationship of these results to the biological effects of xanthydrol, xanthone, and their derivatives is discussed.

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REACTIONS OF XANTHYDROL: II. INSULIN, LYSOZYME, AND RIBONUCLEASE
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