Effect of iodine on the biochemical and immunochemical properties of the toxic lecithinase of clostridium hemolyticum
by Edwin T Parmelee

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Chemistry
Montana State University


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Therefore unaltered tyrosine as an antigenic determinant appears to be ruled out, while the necessity of it's presence for the lethality and leci- thinase activity of the toxin molecule is established.

# EFFECT OF IODINE ON THE BIOCHEMICAL AND IMMUNOCHEMICAL PROPERTIES OF THE TOXIC LECITHINASE OF CLOSTRIDIUM HEMOLYTICUM

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A THESIS

Submitted to the Graduate Faculty

in

partial fulfillment of the requirements

for the degree of

Master of Science in Chemistry

at

Montana State College

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Bozeman, Montana
August, 1949

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## I. ABSTRACT

The presence of tyrosine residues in the toxic enzyme molecules elaborated by Clostridium hemolyticum has been established. Conditions for the rapid toxoiding of this toxin by means of iodine were determined. Under these conditions, the tyrosine groups were completely converted to di-iodotyrosine groups as evidenced by complete abolition of the Millon reaction. Loss of the free tyrosine groups resulted in the destruction of 98.5% of the lecithinase activity of the original toxin, thus establishing the essentiality of free tyrosine groups for the lecithinase activity of this toxin. Moreover, iodine-treated toxin showed a greatly diminished lethal power as evidenced by animal inoculation tests, but antigenicity was retained. Rabbit antibody to the iodinated antigen exhibited a definite tendency to cross-react with the untreated antigen.

Therefore unaltered tyrosine as an antigenic determinant appears to be ruled out, while the necessity of its presence for the lethality and lecithinase activity of the toxin molecule is established.

## II. INTRODUCTION

The study of the products of bacterial metabolism has been of sufficient interest and importance to engage the attentions of workers for at least the last half-century. Some recognition of the chemical nature of these materials has, however, been only of relatively recent origin and this has been particularly true of the soluble exotoxin of the causative organism of bacillary hemoglobinuria (redwater disease) which was first completely described by Records and Vawter (34).

The ground work on this toxin in Montana has been carried out chiefly by Jasmin (21) and by Swingle (37). The main effort has been directed toward producing a vaccinal product that would confer an immunity of longer duration than has previously been attained. Up to the present time such efforts have met with little success; probably, because of the low titer of the culture filtrates with respect to lethal and lecithinase activity. It has been recognized for some time now that a large degree of purification of the toxic material present in the culture filtrates was a logical prerequisite to the further study of the toxin, from both the practical and the theoretical standpoints. Recently, such a purification, amounting to around a thousand fold, has been accomplished (37). The ability to secure the toxic material in such relatively pure form has paved the way for the work to be described here.

In brief, this investigation has consisted of an attempt to determine the presence and essentiality of free tyrosine residues in this toxic enzyme molecule from the standpoint of:

1. Lecithinase activity
2. Lethal activity
3. Antigenic activity

4. Serological cross reactivity

In view of the generally considered high specificity of iodine as a group reagent under proper conditions and the non-specific or, at least, uncertain specificity of other chemical reagents on tyrosine groups (31, 1) it was decided to employ iodine for the study outlined above.

It has been demonstrated (15, 19, 25, 26) that at alkaline pH's the oxidative proclivities of iodine are almost completely excluded in favor of substitution on the aromatic nucleus of the tyrosine residues. The reaction is considered to be the following:

The entrance of iodine at the ortho-positions is to be expected in view of the directive influence of the highly reactive hydroxyl group due to the high electron density of the ortho-positions coupled with the electrophilic nature of the iodine atom.

The choice of iodine as a reagent in this study was further mediated by reason of its rather mild effect on proteins. That is, it does not hydrolyze peptide bonds nor disulfide groups of the protein backbone nor does it usually cause disruption of the original configuration to bring about the often irreversible structural changes known as "denaturation" (31).

Thus, the employment of iodine should enable investigation of the effects of the alteration of a specific group on the biological activity of the protein. If, by means of this reagent, the biological activity could be destroyed or altered, then it should be correct to assume that

the specific group of the protein attacked by this reagent was responsible or played an important part in mediating the activity.

A determination of precisely those groups on a protein molecule responsible for its distinctive biological effects could lead to clues on the structure of such protein materials as viruses, hormones, antigens and, indeed, enzymes such as the one studied here. However, in addition to the large theoretical interest in such matters, there are certain practical considerations. In the present study, there is justification for an attempt at modification of the undesirable properties of this lecithinase so that it may be employed without harm or danger medicinally, i.e., as a toxoid or vaccine. The results of such treatment on toxins like tetanus and diphtheria speak well for attempts to employ a similar means of detoxification. The substance used for this procedure in the case of these toxins, and certain others, has been formaldehyde. However, the use of formaldehyde in detoxifying this lecithinase has always resulted in complete loss of antigenic activity. Some other agent and method for accomplishing this purpose was thus indicated, and iodine was chosen for the reasons previously given.

## III. HISTORICAL

In 1939, the disease of cattle and sheep known as bacillary hemoglobinuria or redwater disease was first recognized in Montana. However, it is known that it was prevalent in the irrigated valleys of western Nevada and the bordering areas of California at least sixty years ago, although there appears to have been some confusion regarding its distinction from anthrax. In 1914, losses amounting to 25 per cent in some herds in the Carson Valley of Nevada were responsible for the institution of a definite research project at the University of Nevada. Initial progress was slow but the work on clostridial forms during and after the first world war brought new techniques applicable to the problems posed by the extreme fastidiousness of the etiological agent of this disease. The organism was grown, the clinical symptoms resulting from its metabolism catalogued, and its bacteriology recorded. Vaccines of various types were prepared, such as phenol-killed whole culture bacterins and glycerinated non-toxic live culture vaccines (40). Protection was conferred for periods up to eight months, perhaps longer. Later the same workers (54) developed an alum-precipitated formalized bacterin which was apparently effective for about a year. The above materials were often accompanied on injection by extraneous skin lesions, non-specific protein reactions and even death of the animal. The cause of death was not always attributable to the toxin since characteristic symptoms were not always present.

More recent work in Montana has been directed along the lines of securing a purified potent toxin for study. Only very recently has this goal been realized to any degree and, therefore, the study in the present paper

represents the first known investigation of the effect of a specific protein group reagent on the lethal, enzymic, and antigenic activities of the purified toxin.

A great deal of work has, however, been done on the crude toxin (37). Such factors as its stability were studied; rapid means and optimal conditions for assay were established; and attempts were made to develop synthetic or at least "semi"-synthetic media. Included also, were singularly unsuccessful attempts to obtain purified toxin, free of non-protein nitrogen, by means of fractional alcohol precipitation. To date, the most effective means of purification involves precipitation employing ammonium sulfate and nucleic acid. The impurities to be expected in the resultant toxic protein are chiefly, then, inorganic (ammonium sulfate) and organic (protamine nucleate) salts and certain proteins, proteoses and peptones which are carried along in the purification process.

## IV. STATEMENT OF PROBLEM

In this investigation an attempt is made to establish or determine:

1. The presence and essentiality of tyrosine groups for the lethal, lecithin-splitting and antigenic activity of the exotoxin elaborated by the anaerobic spore-forming organism Clostridium hemolyticum, the etiologic agent for redwater disease of cattle.

2. The conditions under which this toxin can be iodinated with ease and rapidity without denaturation of the highly labile protein molecule.

3. The effect of the specific protein group reagent, iodine, on the tyrosine groups, provided their presence is demonstrated.

4. Evidence of the ability or inability of the iodinated toxin to produce on injection, antibodies which would cross-react with the original unaltered toxic protein molecule. Also the production of an antigen which would confer a more lasting immunity.

## V. EXPERIMENTAL

### A. Materials and Reagents

#### 1. Iodination Studies

a. Dry purified toxin preparations (37)

1. Toxin KFS III 235 containing 28.5 MLD/mg.

2. Toxin KFS III 248 containing 34.5 MLD/mg.

3. Toxin KFS III 250 containing 75.0 MLD/mg.

b. Purified toxin solutions

These solutions were made up from time to time with distilled water. The pH was adjusted to 7.4 - 7.6 and they were then placed in amber bottles and stored in the refrigerator.

c. Concentrated stock iodine solution

200 ml. of 0.1 N resublimed iodine in 0.5 N potassium iodide were made up and stored in an amber bottle at room temperature.

d. Concentrated stock sodium thiosulfate solution

200 ml. of 0.2 N sodium thiosulfate solution were made up with freshly boiled water and preserved with 0.2 g. of sodium carbonate.

e. Starch indicator solution

A 1 per cent solution of soluble starch was prepared and 3 per cent boric acid added as a preservative.

#### 2. General Reagents

a. Millon's tyrosine reagent

b. Lugg's tyrosine-tryptophane reagent (5)

#### 3. Lecithinase inhibition experiments

a. Egg-yolk substrate (37)

b. Isotonic borate buffer of the following composition: 0.9% NaCl, 0.02 M $CaCl_2$, 0.1 M $H_3BO_3$ and NaOH to pH 7.4.

B. Methods

1. Qualitative test for presence of tyrosine groups in the toxin molecule.

A small spatulaful of dried toxin was dissolved in about 1 ml. of distilled water and two to three drops of Millon's reagent added. The solution was heated very slowly and after several minutes a bright red coloration appeared. The experiment was repeated twice more with the same results, thus establishing the presence of tyrosine in the toxic lecithinase preparation. All remaining work was based on the established presence of "exposed" tyrosine residues in the toxin molecule.

2. Technique of iodination of the tyrosine groups in the toxin molecule.

570 mg. of toxin KFS III 248 were weighed out and placed in about 5 ml. of water, where solution was essentially complete, and the pH was adjusted to 7.4 with dilute NaOH. 13.1 ml. of 0.03 N $I_2$, pH 7.4, were added, and the pH was readjusted to 7.4. (The pH had a tendency to fall slowly with time and had to be brought to 7.4 occasionally with dilute NaOH.) Addition of the iodine was accompanied by the development of some turbidity and a chalky yellowish-brown color. Exactly ten minutes was allotted for entrance of iodine into the ortho-positions of the tyrosine residues. At the end of this time, 0.5 ml. of starch indicator was added and sufficient $Na_2S_2O_3$ run in to completely destroy the slight excess of free iodine. The pH was readjusted to 7.4, (it rose on addition of the thiosulfate), the

solution was quantitatively transferred to a 25 ml. volumetric flask, and borate buffer at pH 7.4 was added to the mark. The mixture was then filtered through a sintered glass (Corning U.F.) filter. Filtration was exceedingly slow in spite of the alkaline pH. There were a few small insoluble particles and a slight turbidity suggesting that the iodine had behaved in part as a precipitant. A qualitative lecithinase test on a portion of the unfilterable material showed that there was some residual activity.

The toxin which passed the filter was a light-brown clear liquid. This material was transferred aseptically to a sterile serum bottle, fitted with a sterile rubber serum-bottle cap, and placed in the refrigerator. This iodo-toxin was labelled Solution II.

B. Demonstration of uptake of iodine by the toxic protein.

Initial experiments designed to show a loss of free iodine in a system containing, in addition to the free iodine, the toxic lecithinase, gave extremely variable results. The toxin solution was diluted to the 2 MLD/ml. level. 2.0 ml. of 0.01 N iodine were allowed to react with 10 ml. of the toxin solution at room temperature for a period of ten minutes. At the end of the time allotted, the excess iodine was titrated with 0.0022 N thiosulfate. However, as stated, the results were erratic. It was therefore felt that the inability to demonstrate conclusively iodine uptake by the protein might have been due in part to the relatively small amount of protein employed (0.07 mg. protein per ml.), so that the limits of accuracy of the method of titration were exceeded. Accordingly a much more concentrated toxin solution was used in the following work.

In this attempt, a toxin solution containing 2 mg. toxic protein per

ml. of solution was employed. The results are shown in Table I.

Table I

Uptake of Iodine by the Toxic Protein

| Toxin | Iodine* added | Thiosulfate** added to titrate excess iodine |
|---|---|---|
| mg. | ml. | ml. |
| 0 | 30 | 29.12 |
| 10 | 30 | 26.25 |

* 0.005 N
** 0.0052 N
These results are the averages of three determinations.

In these trials there was, after several minutes, a very noticeable decrease in the intensity of the yellowish-orange iodine coloration in those flasks containing the enzyme, and a marked decrease after ten minutes, indicating loss of free iodine from solution. This interesting result was borne out in the course of the thiosulfate titrations. That tyrosine groups were involved in the disappearance of free iodine was demonstrated by the complete inability of the iodinated protein to furnish a positive Millon reaction, whereas the untreated toxin produced an excellent test for tyrosine with this reagent.

4. Analytical tests for lecithinase after iodination. Percentage inactivation of lecithinase by iodine.

Initial experiments designed to estimate the amount of residual enzyme activity of the iodinated toxin, designated Solution II, indicated that some activity did remain and that it was only a small fraction of the original. It further provided information as to the region in

which a quantitative lecithinase determination might profitably be run. Accordingly, the following procedure was carried out: 3 ml. of Solution II were diluted 1:6, and each of three dilutions of this solution was assayed in triplicate. The results are recorded in Table II. Since the average number of MLD's in 1 ml. of 1:6 dilution of Solution II was 1.22, a 6 ml. aliquot, representing 1 ml. of the concentrated iodo-toxin contains 6 x 1.22 = 7.32 MLD. Since the toxin solution prior to iodination contained $\frac{370 \times 34.5}{25} = 511$ MLD/ml., the

$$\text{per cent inactivation} = \frac{511.0 - 7.32}{511} \times 100 = 98.5$$

Although the extent of inactivation of lecithinase was 98.5 per cent, it cannot be stated with certainty that it was due exclusively to the iodination process alone.

5. Time study of the effect of iodine on lecithinase activity.

Since it seemed advisable to establish the mildest conditions under which the toxic enzyme could be inactivated, the effect of time of contact of the toxin with a given normality of iodine on the lecithinase activity was studied.

10 mg. of toxin KFS III 235 containing 28.5 MLD/mg. were dissolved in 142.5 ml. of distilled water and the pH adjusted to 7.4 - 7.6 with several drops of dilute NaOH. The potency, therefore, was around 2 MLD/ml. To 5 ml. portions of this solution a constant amount (0.1 ml.) of 0.1 N iodine was added, and, after varying time intervals, reduced with dilute (0.0066 N) sodium thiosulfate using starch indicator to determine the end-point. 2 ml. aliquots of the mixture were then removed and analyzed for lecithinase. The results are shown in Figure 1, where time of contact with