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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Loss of the free tyrosine groups resulted in the destruction of 98.5 of the lecithinase activity of the original toxin, thus establishing the essen-tiality 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. Rab-bit. 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 it's presence for the lethality and leci- thinase activity of the toxin molecule is established.

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EDWIN T. PARMELEE

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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.

370 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 <u>ortho</u>-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

Table II

Determination of Residual Lecithinase Activity
in Toxin Solution II

Tube	Toxin ml.	Buffer ml.	Substrate ml.	Optical Density x 10	MLD/ml.
0	0	5	5	-	-
1	3	2	"	7.30	*
2	"	"	"	7.20	*
3	"	"	"	7.40	*
4	2	3	"	6.80	*
5	"	"	"	6.70	*
6	"	"	"	6.82	*
7	1	4	"	5.00	1.18
8	"	"	"	5.20	1.24
9	"	"	"	5.20	1.24

Substrate J. L. C. 6/13/49 Average 1.22
Buffer E. T. P. 6/7/49
Temp. 37° C.

All readings were made on a Lumetron photoelectric colorimeter equipped with a 650 mu filter. Toxin and substrate were incubated for twenty minutes before reading.

*Optical density readings above 6.2 lie beyond the useful range of the standard curve.

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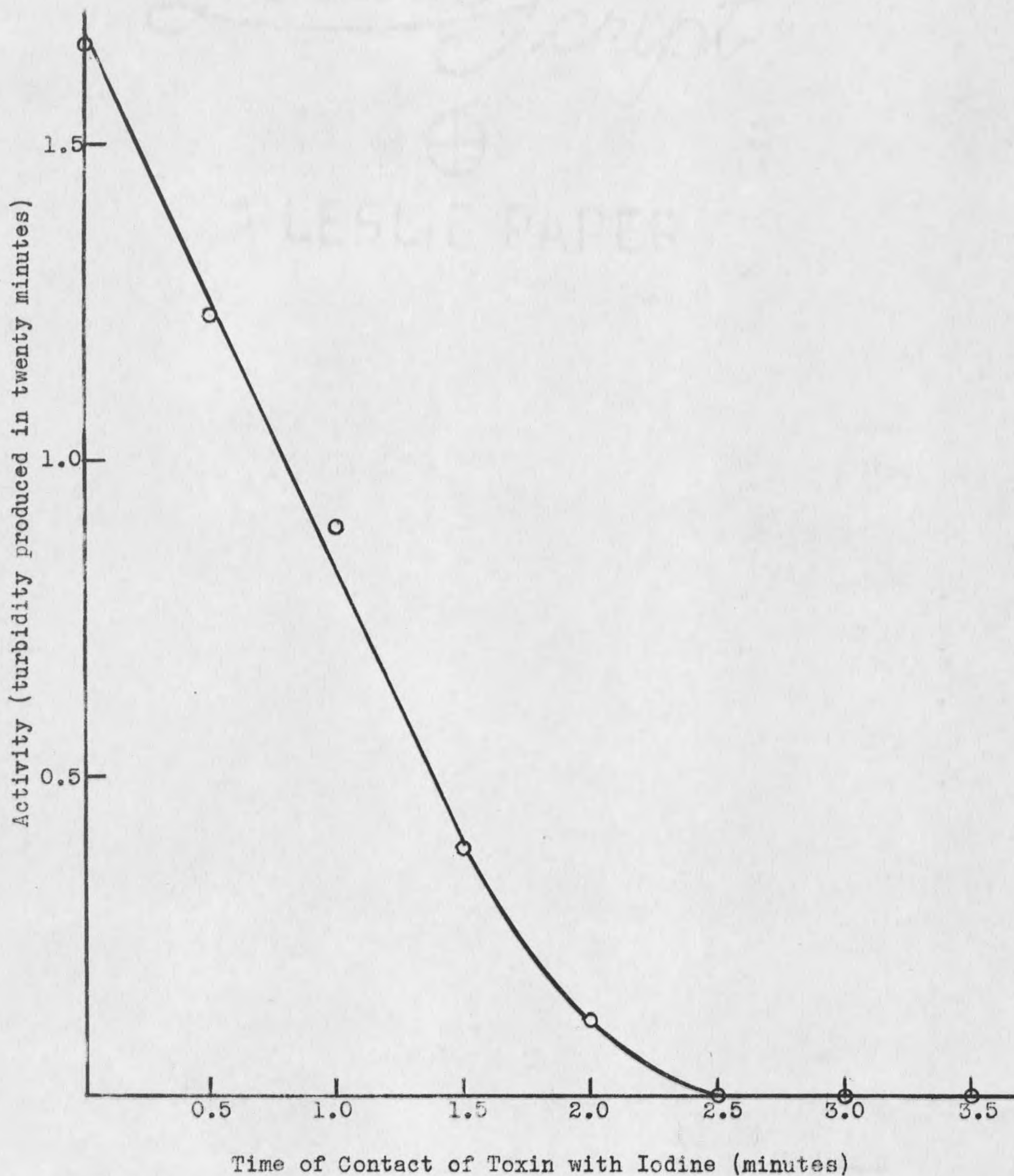

Figure 1. Effect of time of contact of toxin with iodine on lecithinase activity. The ordinate values represent the turbidity produced in twenty minutes, and are read as optical density.

iodine is plotted against optical density of the enzyme-substrate mixture. It may be seen that the iodine rapidly inhibits the ability of the toxic enzyme to cleave its substrate (egg-yolk lecithin) into the fatty acid - diglyceride mixture responsible for the turbidity by which the rate of the reaction is measured. It was discovered in a separate experiment that neither thiosulfate nor starch inhibited the lecithinase reaction; thus, the inhibition represented can be due only to iodine. That it was not actually 100 per cent complete even with 0.1 N iodine for three minutes longer (as might be inferred from Figure 1) should perhaps be mentioned. This curve is the result of the standard assay procedure, according to which turbidity measurements are made after an enzyme-substrate incubation period of twenty minutes. Thus, turbidimetric determinations made after an incubation period of twenty-one hours revealed that some "residual" lecithinase was apparently present.

6. Minimal amount of iodine to inactivate lecithinase.

Determination of the mildest conditions for iodination involved an examination of the minimal quantity of iodine which in a given short interval of time, would abolish lecithinase action in terms, again, of the standard assay procedure.

5 ml. portions of toxin KFS III 235, containing 2 MLD/ml., as before, were placed in a series of tubes. Iodine solutions of graded normalities (0.1 N - 0.00009 N) and at pH 7.4 - 7.6 were added to the tubes and the excess free iodine reduced exactly ten minutes after addition by means of a few drops of 0.2 N thiosulfate. 2 ml. aliquots from each tube were combined with 3 ml. of borate buffer (pH 7.4), 5 ml. of egg substrate added

Table III

Effect of Decreasing Iodine Concentration
on the Lecithinase Reaction

Normality of Iodine	Optical Density			
	20 min. (std.)	1 hour	5 hours	40 hours
0.0 (control)	5.90	7.50	7.50	7.50
0.1	0.00			1.36
0.08	0.00			2.03
0.06	0.00			2.45
0.04	0.00			3.00
0.02	0.00			
0.009	0.00			
0.007	0.00			
0.005	0.00			
0.003	0.00	1.03		
0.002	0.05	1.12	1.76	
0.001	0.10	1.45	2.35	
0.0009	0.28		2.43	
0.0008	0.65			
0.0007	0.75			
0.0006	1.02			
0.0005	1.25		3.78	
0.0004	1.86		4.90	
0.0002	2.50		6.40	
0.0001	3.82		7.08	
0.00009	5.90		7.50	

and the mixture assayed for lecithinase. The analytical results are shown in Table III. In the table, the 20 minute assay results cover the entire range of iodine concentrations, while those assays made after incubation of enzyme-substrate for 1, 5, and 40 hours were selected so as to indicate simply the trend at these later periods. It may be noticed that in no case was the lecithinase reaction of the iodine-treated toxin as rapidly completed as that of the non-iodinated control - with the one exception in which 0.00009 N iodine was employed. An interesting situation was observed in the tubes containing toxin solutions treated with less than 0.0005 N iodine. In these tubes, after ten minutes, there was no development of a blue coloration upon the addition of starch indicator. It would thus appear that all of the iodine added had been taken up by the toxin. This fact was the basis for the establishment of 1 ml. of 0.0005 N iodine as the least amount of iodine which in ten minutes time completely iodinated all of the tyrosine groups of the toxin preparation employed. Figure 2 shows the manner in which the enzyme activity responded to increments in iodine concentration.

7. Mouse tests of lethal action of toxin after iodine treatment.

Mature, white, inbred mice weighing 20 - 25 grams were used in all tests of the lethality of the iodinated toxin. Sterile dilutions of two separate iodinated toxin preparations were made in the following manner:

Small samples of the sterile concentrated iodo-toxin were withdrawn from the serum-bottle with sterile syringes and transferred to several sterile serum bottles. To these samples, sterile isotonic borate buffer was added in sufficient amount to yield dilutions of the concentrated ma-

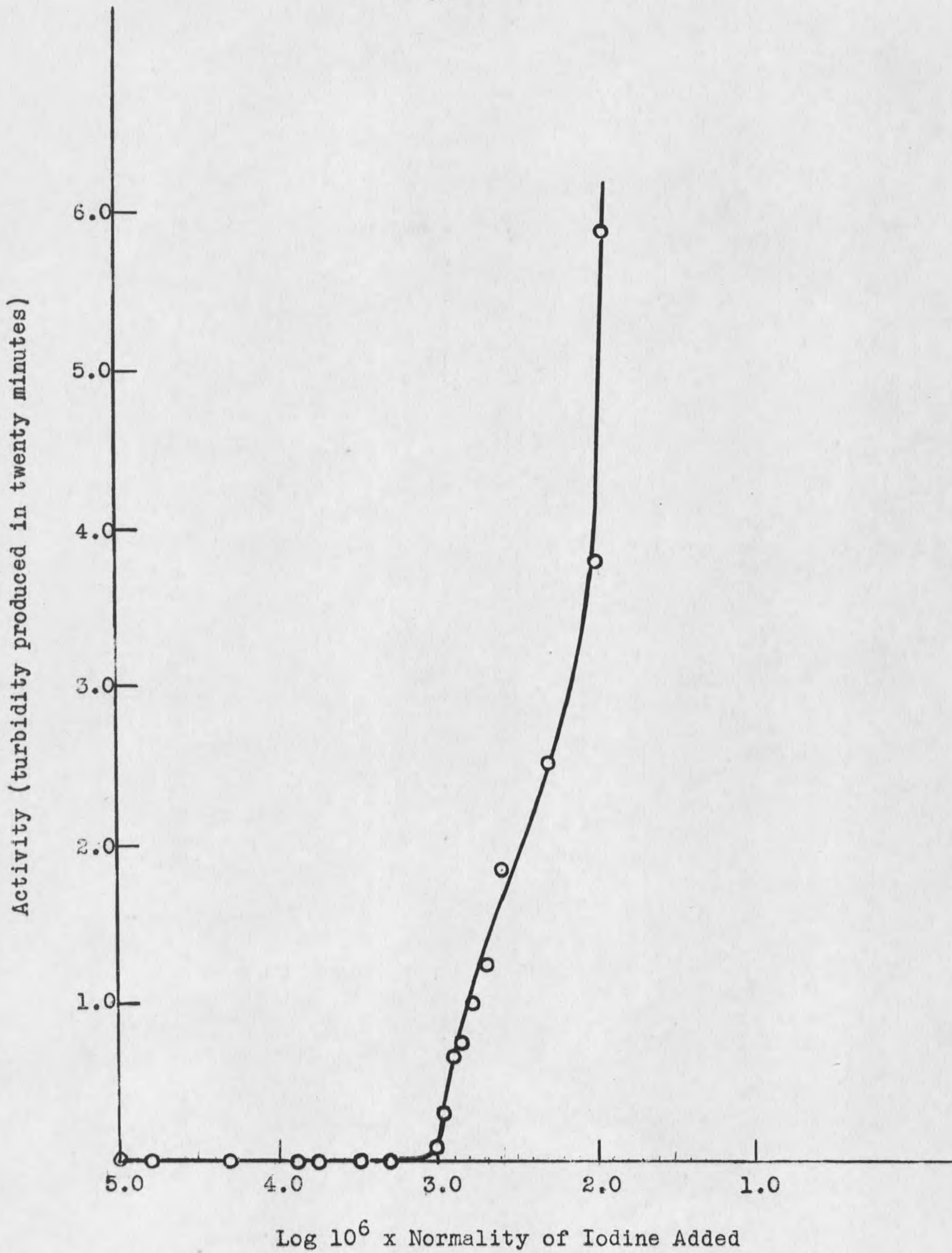

Figure 2. Effect of concentration of iodine on lecithinase activity.

terial of 28.5, 24, 16, 8, 4, and 2 MLD/ml. The mice were injected intravenously (caudal vein) with 0.5 ml. of this material according to the scheme shown in Table IV.

All mice survived, including those inoculated with a dosage of toxin which before iodination would have been sufficient to kill 28.5 mice.

That both the lethal and lecithinase activities of this toxin are dependent upon the presence of free tyrosine groups would thus seem to be indicated. Such correspondence between the two activities had already been noted elsewhere (21), but was based on other evidence.

8. Technique and course of rabbit inoculations.

An accelerated program of rabbit inoculations was initiated in an attempt to build up as rapidly as possible rabbit immunity to the iodo-toxin. While it would have been desirable to employ several animals in an endeavor to demonstrate an antibody to lecithinase, the use of only one was conditioned by the non-availability of more than a few hundred milligrams of the purified toxic material. Therefore a single, white, female rabbit weighing about 2200 grams was selected for the inoculations. All injections were made subcutaneously using a 21 gauge needle. The iodinated toxin preparation used (Solution II) contained approximately 510 MLD/ml. or 14.8 mg. protein/ml. Table V gives the complete schedule of injections. The final injection of 2550 MLD's would have been sufficient, had it not been treated, to have killed at least twenty normal rabbits of the same age and weight.

9. Tests for presence of antilecithinase in the serum of the injected rabbit.

Table IV

Mouse Tests of Lethal Power of Iodo-lecithinase

Solution	*MLD's/0.5 ml. Injected	Solution Injected ml.	Protein/o.5 ml. mg.	Result
IA	1.0	0.5	0.035	Survived
IB	2.0	0.5	0.07	"
IC	4.0	0.5	0.14	"
ID	8.0	0.5	0.28	"
IE	16.0	0.5	0.56	"
IF	24.0	0.5	0.84	"
I	28.5	0.5	1.00	"
IIA	25.5	0.5	0.74	"

*MLD - The least amount of untreated toxin killing within 24 hours all mice injected. The tabulated values refer to the lethality of the toxin before iodination.

Table V

Schedule of Inoculations
for Production of Rabbit Antilecithinase

Days	Soln. II Injected	MLD's* Injected	Protein Injected	Symptoms
	ml.		mg.	
1st	1.0	510	14.8	moderate anorexia, lethargy
5th	1.5	765	22.2	slight anorexia, lethargy
9th	2.0	1020	29.6	none
13th	2.5	1275	37.0	none
16th	3.0	1530	44.4	none
19th	3.5	1785	51.8	none
22nd	5.0	2550	74.0	none
Totals	18.5	9435	273.8	

* These values refer to mouse MLD's before iodination.

At regular intervals, usually on the same days as the rabbit received injections, 5-7 ml. of blood were obtained by severing, with a sharp scalpel, the marginal ear vein. This proved a rapid and effective means for obtaining non-hemolyzed sera. After separating the serum by centrifugation, several drops of chloroform were added and the serum sample placed in the refrigerator until assayed. (The assays were invariably performed within 24 hours of the time the blood was drawn.)

Antilecithinase determinations were carried out according to the following procedure:

Serum was placed in matched Lumetron tubes in graded amounts (0.01 ml. - 0.5 ml.) and exactly one ml. of purified toxin solution, pH 7.4, containing about 2 MLD/ml. added. Sufficient borate buffer was run in to bring the volume to 5 ml. The toxin-serum-buffer mixture was then incubated for 30 minutes at 37°. At the end of this time, 5 ml. of egg substrate were added to each tube and the assay performed in the usual manner. Control tubes (no serum) were also run at the 2 MLD and 1 MLD levels. The number of milliliters of serum required to reduce the lecithinase value from the 2 MLD to the 1 MLD level was then determined for each of the serum preparations. In a separate experiment it was found that chloroform, in the amounts employed for serum preservation, had no inhibitory effect on the lecithinase reaction. A graph illustrative of the increase in antitoxic titer with time is shown in Figure 3. In order to take into consideration possible change in the stock (2 MLD/ml.) toxin solution used in the antilecithinase assays, the 2 MLD and 1 MLD points on the assay curves were established for each new serum preparation assayed.

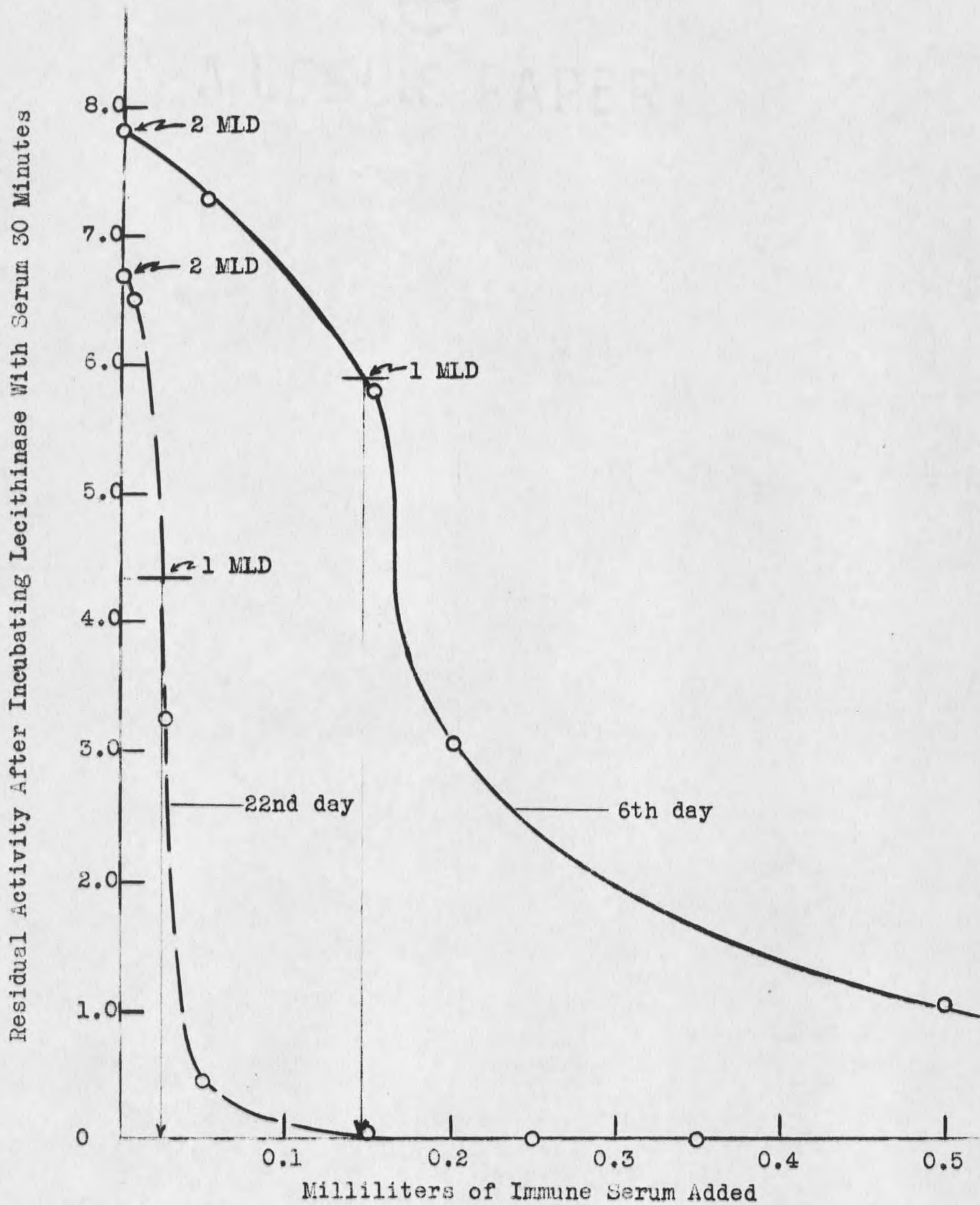

Figure 3. Antilecithinase titer of sera obtained on different days of immunization. The ordinate values represent the turbidity produced in twenty minutes, and are read as optical density.

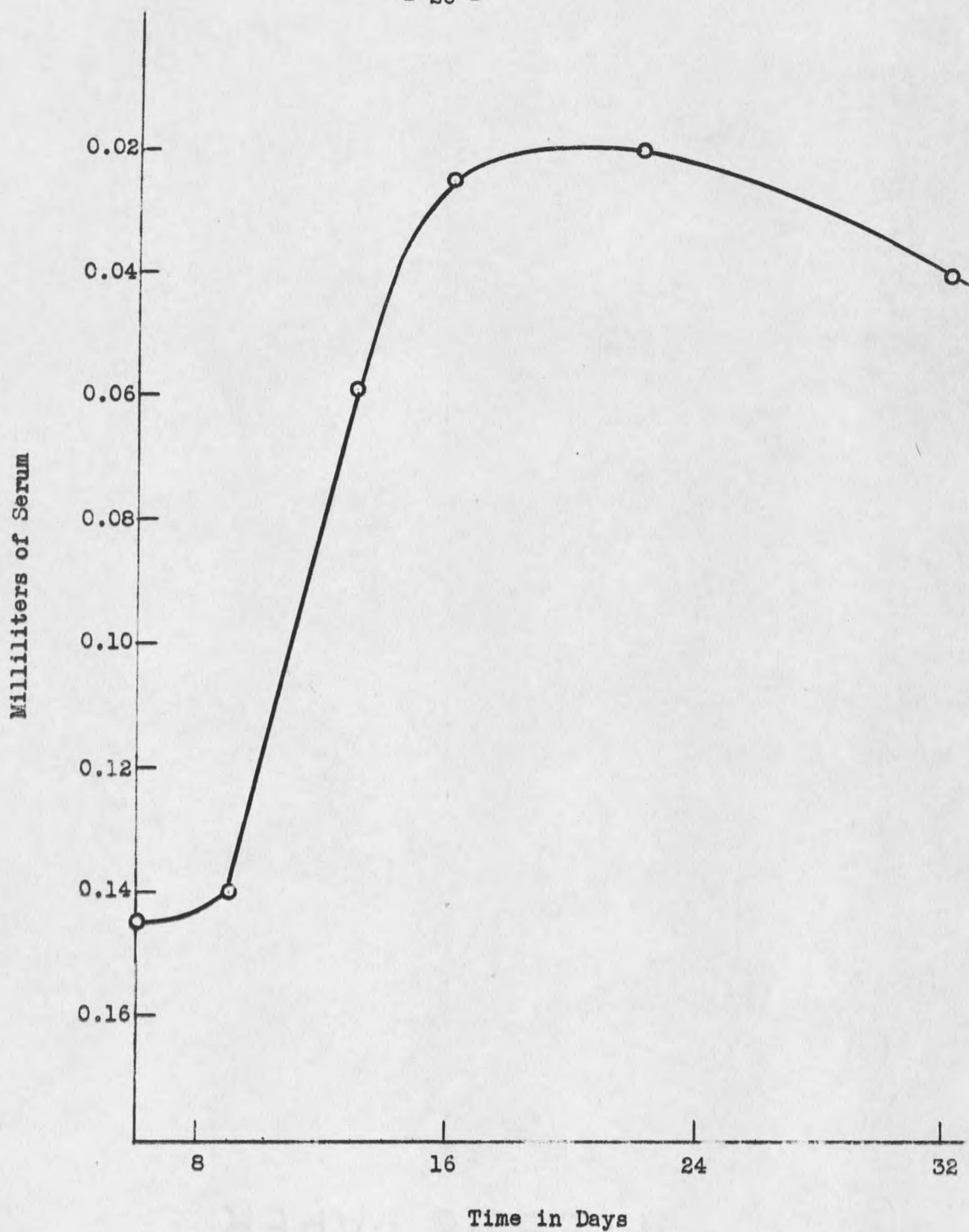

Figure 4. Antilecithinase titer vs. time of immunization. The titer is expressed in terms of milliliters of serum to reduce the activity of 2 MLD toxin to 1 MLD.

Figure 4 indicates the overall results obtained from the course of inoculations described in Table V.

10. Estimation of total tyrosine of the toxin and its relation to iodine uptake.

During the course of this investigation, it appeared that a determination of the total tyrosine present in the purified toxin preparation might yield interesting theoretical results, particularly if a relationship could be established with the iodine uptake of a sample of the same preparation. Under the conditions of iodination employed here, it should be expected that two moles of iodine would disappear for every mole of tyrosine present in the preparation.

Accordingly, Block and Bollings (5) modification of Lugg's procedure (27) for the determination of total tyrosine was employed, with slight variations, for the estimation of tyrosine. The reagents used were made up exactly as described by these authors, except for omission of the color reagent which is not required for tyrosine determinations.

A standard tyrosine solution was prepared by weighing out 25 mg. tyrosine, dissolving in 5 ml. of 7 N sulphuric acid, and adding distilled water to the mark in a 250 ml. volumetric flask (0.1 mg/ml).

Three 30 mg. samples of toxin KFS III 250 (75 MLD/mg.) were weighed into micro-test tubes (5 ml.) and hydrolyzed with 0.65 ml. of 5 N NaOH in an oil bath at 110-125° for 5 hours. After hydrolysis, 1 ml. of 7 N sulphuric acid was added to each sample, the mixtures transferred to 15 ml. centrifuge tubes and water added to the 10 ml. level. A small amount of "Super-cel" (Johns-Manville) was mixed in and the tubes centrifuged a short

time to throw down the gel-like solids. Three aliquots, containing about 0.3 - 0.6 mg. tyrosine each, were removed from each tube. (The assumption was made that the toxic protein contained 5 per cent tyrosine. Therefore, in 10 ml. there would be .05 x 30 = 1.5 mg. tyrosine.)

The method employed from this point on was essentially that of Block. An exception was the allowance of ten minutes, rather than five, for color development after addition of the sodium nitrite reagent. This gave sufficient time for manipulation (transfer of a 10 ml. aliquot to Lumetron tubes, warming, reading, etc.) and it was found that the color did not change in intensity over the additional five minute period.

The standard curve was prepared by carrying the known tyrosine solutions through exactly the same procedure as for the unknowns with the exception of the hydrolysis step. The data are plotted in Figure 5. Table VI gives the experimental values obtained on the unknown samples.

It was found that the percentage tyrosine in this toxin preparation was 2.8. A comparison of the percentage of tyrosine with the uptake of iodine was then attempted. The amount of iodine taken up by 10 mg. of toxin KFS III 250 has already been discussed in Part 3 of the experimental section, and was found to be 2.87 x 1.04 ml. of 0.005 N iodine. The following calculations were therefore made with the data furnished by the two experiments described:

A. Moles of iodine required for complete iodination of 10 mg. toxin.

Per cent tyrosine = 2.8

Grams " per 10 mg. toxin $= 0.28 \times 10^{-3}$

Moles " per 10 mg. toxin $= \dfrac{0.28 \times 10^{-3}}{0.182 \times 10^{3}} = 1.54 \times 10^{-6}$

Moles iodine to completely iodinate 1.54×10^{-6} moles tyrosine $= 2 \times 1.54 \times 10^{-6} = 3.08 \times 10^{-6}$

B. Moles of iodine actually taken up by 10 mg. toxin.

ml. iodine taken up $= 2.96$
equivalents iodine taken up $= 2.96 \times .005 \times 10^{-3} = 1.480 \times 10^{-5}$
moles iodine taken up $= \frac{1.480 \times 10^{-5}}{2} = 7.4 \times 10^{-6}$

It would appear therefore that over twice (2.4 times) as much iodine was taken up by the toxic protein as would be expected on the basis of the percentage of tyrosine found. (See discussion.)

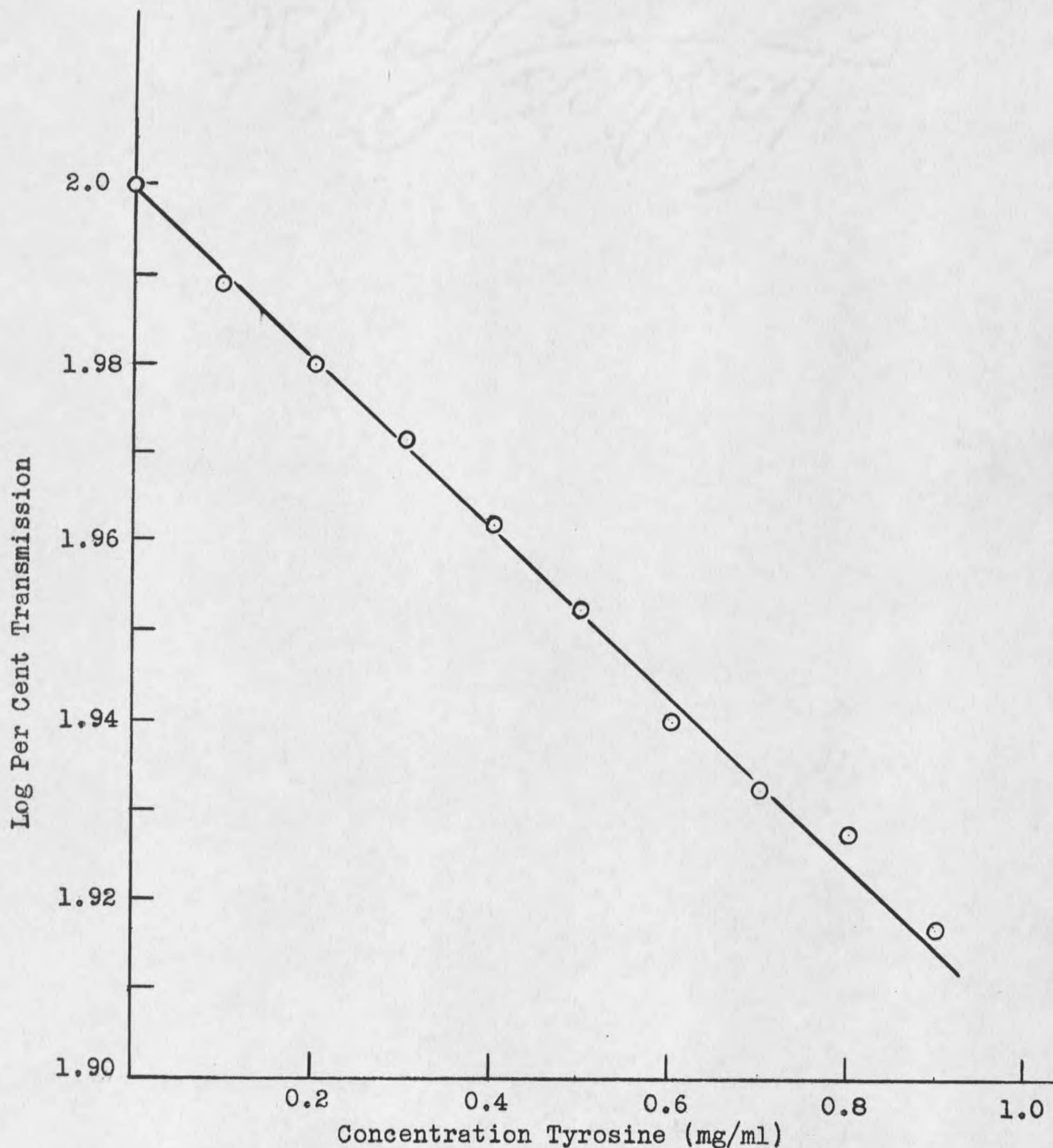

Figure 5. Standard Tyrosine Curve. Readings were taken on a "Lumetron" photoelectric colorimeter using a 520 mu filter.

Table VI

Summary of Experimental Values and Derived Date
for the Determination of Tyrosine on Toxin KFS III 250

Tube	Total wt. Toxin spl.	Toxin/tube	Trans-mission	Trans-mission	Tyrosine per tube	Total tyro. toxin spl.	Total tyro. toxin spl.
	mg.	mg.	%	Log. %	mg.	mg.	%
Blank	0	0	100	2.0	0.00	0.00	0.00
1a	30.6	0.3 x 30.6	95	1.978	0.23	0.77	2.5
1b	30.6	"	94	1.973	0.28	0.93	3.0
1c	30.6	"	94	1.973	0.28	0.93	3.0
					Ave. 0.26	0.88	2.8
2a	31.8	0.3 x 31.8	94.5	1.975	0.26	0.87	2.7
2b	31.8	"	95	1.978	0.23	0.77	2.4
2c	31.8	"	94.8	1.977	0.24	0.80	2.5
					Ave. 0.24	0.81	2.5
3a	30.7	0.3 x 30.7	94.5	1.975	0.26	0.87	2.8
3b	30.7	"	95	1.978	0.23	0.77	2.5
3c	30.7	"	93	1.968	0.33	1.11	3.6
					Ave. 0.27	0.92	3.0
Average							2.8

The values for tyrosine/tube were read from the standard tyrosine curve shown in Figure 5.

VI. DISCUSSION OF RESULTS

The importance of unaltered tyrosine for the biological activity of the vast majority of proteins studied has been noted by Olcott (51). However, little, if any, notice has been paid the reasons for the importance of this amino acid in hormones, viruses, enzymes and the like. It is suggested that the open ortho-positions of the unaltered tyrosine residue in conjunction with the activity conferred by the reactive hydroxyl group might account for the ability of the larger, unaltered protein molecule to enter into two types of combinations in the animal body:

1. Those which are of benefit to the elaborating organism, be it the host or invader, or

2. Reactions (such as the one catalyzed by the toxic lecithinase) which are of severely harmful consequence to the host.

If, as would appear indicated by the results of this work, unaltered tyrosine is essential to the second of these types (in this case), then chemical blocking of the protein groupings necessary for such a reaction could yield a material tolerable to the foreign animal, and yet possessing those qualities required to elicit neutralizing substances when injected into the tissues or blood stream of the animal.

The question of whether the antibodies produced in response to injections of iodo-lecithinase are the result of the remaining 1.5 per cent lecithinase activity or are chiefly antibodies to the iodo-antigen has not been answered completely. There are several possible approaches to this problem, however. For example, the serum of animals inoculated with the number of MLD's of untreated purified toxin KFS III 248 equivalent to 1.5 per

cent of its original activity could be assayed over a course of immunization to determine whether the antitoxic titer responded in the same manner as with the iodinated toxin.

Inhibition of the antibody to the iodinated antigen by means of diiodotyrosine would be further evidence that the antilecithinase power of the serum was indeed antibody to the iodo-protein and not simply a response to the small amount of unaltered material which remained.

Finally, restoration of part or all of the original activity of the toxin by removal of the iodine would be of great aid in resolving this question. That catalytic dehalogenation of a protein is feasible was demonstrated by Harington et al (11) when they dehalogenated iodinated insulin, thereby restoring 30-50 per cent of its original activity.

The finding that around 2.4 times the amount of iodine had been taken up by the toxin as was theoretically possible on the basis of the calculated tyrosine content was not confirmed. That this result may not have been due entirely to experimental error receives some support from the work of Bauer and Strauss, quoted by Herriott (19) , who found that globin (from hemoglobin), which is rich in histidine, absorbed much more iodine than expected from the tyrosine analyses. Likewise, Pauly, quoted by Herriott (19), found that imidazole and the imidazole nitrogen of benzoyl histidine react with iodine. In the latter compound, two iodines substituted. In addition, Shahrokh (35) observed considerable oxidation during iodination of crystalline serum albumin at pH 6.5. There is, in the present case, some possibility that too great an excess of iodine was used in the experiemnt designed to show iodine uptake by toxin KFS III 250. Under such conditions, it may

be that some oxidation (of SH) and substitution into histidine could have occurred. In order that such effects might be minimized it is suggested that iodination of any protein on which the effects of such treatment are being studied be preceeded by a total tyrosine analysis. The stoichiometric amount of iodine for complete iodination of the tyrosine alone could then be added. In addition, a determination of the iodine content of reprecipitated iodo-toxin could be used as further evidence that only the tyrosines had been substituted and that no oxidation had occurred. Determinations of iodine in proteins have been described by Herriott (16, 17) and by Stimmel and McCullough (36).

Among those workers who have, however, been able to demonstrate a close correspondence between iodine uptake and tyrosine content are Li (26), who iodinated lactogenic hormone and Neuberger (30), who carried out studies on iodinated zein. Likewise, Kleczkowski, quoted by Boyd (7), iodinated horse serum globulin and found that the amount of iodine which entered the molecule was accounted for by the tyrosine groups. However, it is interesting that the latter worker discovered that iodination destroyed the ability of his protein to produce antibodies to native horse globulin.

It would seem that iodination of this toxic protein provides a practical means for altering (toxoiding) the lecithinase toxin of Clostridium hemolyticum to yield a material which may be injected safely in large amounts in order that antibody can be produced which is capable of cross-reacting with the original toxin.

VII. SUMMARY

1. The amino acid tyrosine was shown to be present in the lethal toxin of <u>Clostridium hemolyticum</u>, an enzyme catalyzing the hydrolysis of lecithin.

2. Chemical alteration of the free tyrosine groups by complete conversion to di-iodotyrosine destroys 98.5 per cent of the original activity of the toxin. Completeness of conversion to di-iodotyrosine was shown by the inability of the toxin to furnish a positive Millon's reaction.

3. The shortest time of contact with iodine and the lowest concentration of iodine necessary for complete iodination were determined.

4. The techniques of iodination, sterilization, etc., of the purified toxin are described.

5. Animal tests of the lethal power of iodo-lecithinase and the technique and course of rabbit immunization to the toxin are presented.

6. Experiments designed to demonstrate the uptake of iodine by the toxin and its quantitative relationship to the tyrosine analysis of the purified toxin are presented and discussed. It was concluded that the results obtained should not be accepted unconditionally since they have not been confirmed.

7. Evidence is given of the greatly diminished lethal power of the toxin after iodination with, however, retention of antigenicity.

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