STUDIES ON PEROXIDATIVE DETOXIFICATION OF PURIFIED DIPHTHERIA TOXIN

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It has been shown previously that purified myeloperoxidase[1] and crystalline horseradish peroxidase detoxify diphtheria and tetanus toxin when mixtures of crude toxin and peroxidase are dialyzed against dilute hydrogen peroxide (4). Toxin preparations treated in this way can be injected into guinea pigs in amounts corresponding to more than 100 times the lethal dose without evidence of toxic symptoms. No detoxification occurs when the toxins are treated with hydrogen peroxide or peroxidase alone. The toxins used in these previous experiments were produced on a peptone-containing medium and had not been subjected to any purification.

In an attempt to determine the mechanism of peroxidative detoxification of diphtheria toxin, the previous experiments have been repeated using highly purified preparations of toxin. It has been found that purified diphtheria toxin is not detoxified by peroxidase and hydrogen peroxide unless one or more dialyzable, oxidizable substances present in the original medium are added. Dialyzable "cofactors" for the peroxidative detoxification of toxin have also been found in acid-hydrolyzed casein, urine, and other materials (5, 6). Peroxidation of diphtheria toxin in the presence of material containing dialyzable, oxidizable cofactors, yields a colored product which has lost up to 99 per cent toxicity without loss of flocculation titer. The modified toxic protein thus possesses all the properties of a toxoid.

Methods and Materials

Cultural Methods.—The Park-Williams No. 8 (Toronto) strain of *Corynebacterium diphtheriae* was grown on the casein hydrolysate medium described by Mueller and Miller (7). Optimal toxin production was obtained by addition of 0.2 mg. $FeSO_4 \cdot 7H_2O$ per liter and of

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[1] The peroxidase, isolated in 1941 from leucocytes, was originally termed verdoperoxidase because of its green color (1). Theorell and his coworkers have since isolated another brown-green peroxidase from milk (lactoperoxidase, 2, 3). In order to distinguish between these two "verdoperoxidases," it has been suggested that the name of the peroxidase from leucocytes be changed to myeloperoxidase (2).

maltose and glucose up to final concentrations of 1.1 and 0.2 per cent respectively. Flasks were incubated 6 days at 35°C. after which the cultures were filtered. From 10 liters of medium, 8.8 liters of toxic filtrate was harvested containing 65 Lf/cc. The final pH was 7.4.

Purification of Toxin; Step 1.—8.8 liters of toxic filtrate containing a total of 570,000 Lf units was dialyzed for 72 hours against saturated ammonium sulfate solution, containing an excess of solid ammonium sulfate. The pH was maintained at 6.8 throughout by periodic addition of ammonia. The precipitate which formed on dialysis was collected by centrifugation and dissolved in M/10 phosphate buffer. Volume: 750 ml.; activity: 700 Lf/ml.; yield: 90 per cent.

Subsequent purification steps were performed at room temperature (22–25°C.).

Step 2.—550 ml. of saturated ammonium sulfate solution, adjusted to pH 6.8 with ammonia, was added to the solution obtained from step 1. The precipitate was discarded and the ammonium sulfate concentration of the supernatant was brought to 65 per cent saturation. The precipitate was collected by centrifugation and dissolved in distilled water. Volume: 350 ml.; activity: 1250 Lf/ml.; yield: 85 per cent.

Step 3.—4 volumes of saturated magnesium sulfate solution, pH 6.1, was added. The precipitate was discarded and the supernatant saturated with sodium sulfate. The solution was filtered and the precipitate dissolved in distilled water and dialyzed against phosphate buffer, 0.1 M pH 6.8. Volume: 270 ml.; activity: 1200 Lf/ml.; yield: 75 per cent. The solution contained 1650 Lf/mg. of nitrogen and in a concentration of 1 per cent was yellow-red in color.

Up to this point 330,000 Lf units out of the starting 510,000 had been recovered, corresponding to an over-all yield of 58 per cent.

The solution from step 3 was subjected to further fractionation with ammonium sulfate. The ammonium sulfate concentration was first adjusted to 45 per cent saturation by adding 220 ml. saturated ammonium sulfate solution at pH 6.2. The ammonium sulfate concentration was then increased stepwise to 50, 55, and 65 per cent saturation. The precipitates obtained at each of the above ammonium sulfate concentrations were dissolved in 0.1 M, pH 6.8 phosphate buffer, dialyzed until free from ammonium sulfate, and diluted to a concentration of 1000 Lf/ml. Each of these four factions was fractionated once more by adding ammonium sulfate in successive amounts, as described above. Small amounts of material of higher as well as of lower specific activity could be isolated from each of the four fractions. The main portion from each fraction, however, had almost the same properties as its parent fraction. The amount of toxin in each of the four parent fractions expressed in Lf units, the number of Lf units per milligram of nitrogen, the Kf_{60} value,[2] and the number of M.L.D. per Lf are summarized in Table I.

Properties of Purified Toxin Fractions.—The toxin which precipitated between 55 and 65 per cent saturation (fraction 4) had a very faint yellow color in 1 per cent solution. The spectrum for a solution of fraction 4 containing 1 mg. nitrogen per ml. is shown in Fig. 1. The dotted line in this figure represents the light absorption of 3.3×10^{-3} molar tyrosine, an amount which corresponds to the calculated value for the tryrosine content of the diphtheria toxin solution assuming the values for nitrogen and tyrosine content, of 16.0 and 9.5 per cent respectively, given by Pappenheimer (8). About 98 per cent of this preparation moved as a single component upon electrophoresis in a pH 7.10 phosphate buffer of ionic strength 0.1 (Fig. 2). The electrophoretic mobility in this buffer was 5.2×10^{-5} cm.$^2 \times$ volt$^{-1} \times$ sec.$^{-1}$. 99 per cent of the protein nitrogen of fraction 4 was specifically precipitable by antitoxin.[3] The toxin in 0.7 per cent solution was stable at 0–2°C. under sterile conditions. The Lf value as well as the number of M.L.D. per Lf unit had not changed when tested after standing for 3 months in the cold.

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[2] Kf_{60} denotes flocculation time of 50 Lf/ml. of toxin with antitoxin at 42°C.

[3] We are indebted to Dr. Melvin Cohn for determining the per cent of toxin specifically precipitable by antitoxin.

All the toxin preparations isolated at lower ammonium sulfate concentrations were yellow-red in color and showed higher light absorption per milligram of nitrogen in the wave length region above 310 mμ. This was especially pronounced in the spectral region between 310 and 400 mμ, where the extinction of fraction 2 was about three times that of fraction 4.

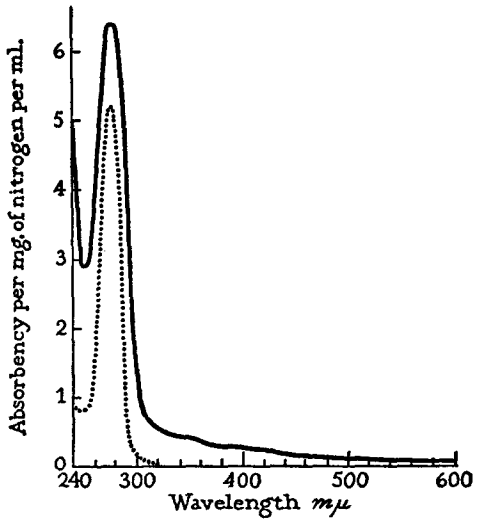

FIG. 1. Absorption spectrum of diphtheria toxin, calculated per milligram of nitrogen per milliliter. The dotted line represents the absorption of 3.3×10^{-3} molar tyrosine.

TABLE I

Properties of Purified Toxic Fractions

Fraction No.	$(NH_4)_2SO_4$ saturation	Toxin precipitated	Specific activity	Kf_{10}*	Toxicity
	per cent	*total Lf units*	*Lf/mg. N*	*min.*	*M.L.D./Lf*
1	45	22,000	800	22	—
2	45–50	90,000	1400	13	15
3	50–55	115,000	1900	11	40
4	55–65	75,000	2200	9	55

* Kf = flocculation time in minutes at 42°C. The subscript denotes flocculation titer in Lf/ml.

The electrophoretic pattern for fraction 2 also differed from that of fraction 4 in that the area representing a component with the same electrophoretic mobility as had been found for fraction 4 comprised less than 30 per cent of the total. The main component of this fraction had a lower electrophoretic mobility (Fig. 3).

The above fractionation procedure has now been applied to two different toxin preparations.[4] Practically the same yield of purified material has been obtained in both cases.

[4] We are indebted to Dr. H. D. Piersma, Lederle Laboratories, and to Dr. Fred Stimpert, Parke, Davis and Company, for providing the fresh toxin for these preparations.

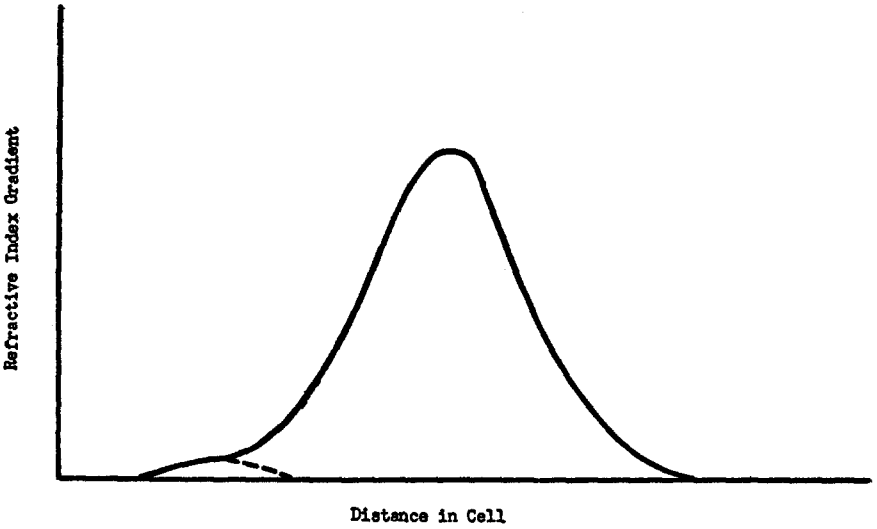

FIG. 2. Descending electrophoretic pattern for diptheria toxin, fraction 4 (ϵ boundary not shown in diagram). Phosphate buffer of pH 7.10 and ionic strength 0.1.

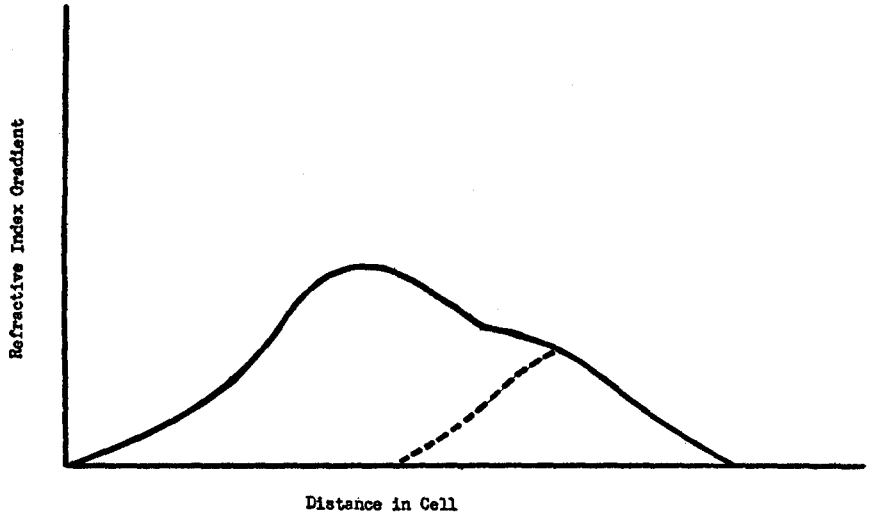

FIG. 3. Descending electrophoretic patterns for diphtheria toxin, fraction 2 (ϵ boundary not shown in diagram). Phosphate buffer of pH 7.10 and ionic strength 0.1.

We have also investigated the yield of purified toxin from a crude toxic filtrate which had been allowed to stand under sterile conditions for 1 month. The amount of material precipitable at lower salt concentrations had increased while the amount of toxin with characteristics corresponding to fraction 4 (Table I) was relatively small.

Peroxidase.—Horseradish peroxidase and myeloperoxidase were prepared according to the methods described by Theorell (9) and by Agner (1).

Detoxification Experiments

Experiments on the peroxidative detoxification of diphtheria toxin were carried out as follows:

Purified diphtheria toxin and peroxidase were placed together in a narrow cellophane bag and dialyzed against a large volume of solution containing the material to be tested for activity as a cofactor. Hydrogen peroxide was added to the solution outside the bag which will be referred to, herafter, as the "dialysate." The pH of the dialysate was maintained at 71. by addition of phosphate buffer.

Addition of hydrogen peroxide to the solution outside the bag assures a continuous supply of H_2O_2, together with the dialyzable cofactors, by diffusion through the cellophane membrane. As noted elsewhere (10) the hydrogen peroxide concentration must be kept at a very low level in order to prevent inhibition or destruction of peroxidase. In most experiments the concentration of H_2O_2 in the dialysate was adjusted to 0.00025 molar. 3 hours after commencing an experiment and at intervals thereafter the peroxide concentration in the dialysate was checked by addition of pyrogallol and peroxidase. If purpurogallin formation was slight or absent, more hydrogen peroxide was added.

Experiments with Casein Hydrolysate

Crude toxic culture filtrates from *Corynebacterium diphtheriae* grown on casein hydrolysate medium are rapidly detoxified by peroxidase in the presence of hydrogen peroxide. The detoxification does not occur if the crude toxic filtrate is first dialyzed free of all substances of low molecular weight or if purified toxin is used in place of crude. It is clear that one or more dialyzable cofactors are required. The following experiments, summarized in Table II, show that the cofactor or cofactors are present in acid-hydrolyzed casein.

In Experiment A, 8 ml. of diphtheria toxin (200 Lf/ml.) and 10 μg. myeloperoxidase were dialyzed against 800 ml. of approximately 1 per cent acid-hydrolyzed casein adjusted to pH 7.1 with phosphate buffer containing H_2O_2 in a concentration of 0.00025 molar. After 24 hours' dialysis, only one of two guinea pigs injected with an amount of peroxidase-treated toxin equivalent to 100 M.L.D. died within 5 days. Experiment B was carried out in exactly the same way as Experiment A, except that the myeloperoxidase was added to the dialysate. No significant loss in toxicity occurred within 24 hours.

In Experiment A, as hydrogen peroxide and the dialyzable substances of casein hydrolysate entered the cellophane membrane, the toxin solution inside became an intense reddish brown. After 24 hours, as noted above, approximately 99 per cent of the toxicity was lost. The flocculation titer, however, remained unchanged at 200 Lf/ml. although the flocculation time (Kf) was increased. Subsequent dialysis of the peroxidase-treated toxin against phosphate buffer failed to remove the reddish-brown color. Moreover, it was noted that the specific floccules formed with antitoxin were also colored. The color

could not be removed from the floccules by repeated washing with saline. It seems clear that the peroxidative detoxification of diphtheria toxin results in the formation of a colored protein with the properties of a toxoid.

In Experiment B in which the peroxidase was added to the solution outside the cellophane membrane, the dialysate became colored almost immediately. The colored oxidized substances which formed, diffused into the bag and after 24 hours the toxin solution was almost as strongly colored as in Experiment A. On subsequent dialysis against phosphate buffer, however, the toxin solution became colorless once more. Moreover, no increase in flocculation time occurred and the toxicity remained undiminished in Experiment B.

TABLE II

Peroxidation of Diphtheria Toxin in the Presence of Hydrolyzed Casein

Experiment A: 8 ml. purified toxin (fraction 4) containing 200 Lf/ml. and 10 μg. myeloperoxidase per ml. was dialyzed at pH 7.1 against 800 cc. of 1 per cent acid-hydrolyzed casein and 0.00025 M H_2O_2.

Experiment B: 8 ml. of toxin containing 200 Lf/ml. was dialyzed at pH 7.1 against 800 cc. of 1 per cent acid-hydrolyzed casein and 0.00025 M H_2O_2 containing 2 μg. myeloperoxidase per ml.

After 24 hours' dialysis, the materials were removed from the dialyzing bags and the M.L.D. determined. Each dilution was injected into two guinea pigs (240 to 270 gm.).

Treated toxin injected per guinea pig	Toxicity	
	Experiment A	Experiment B
Lf units		
10	2 died, 2 days	—
3	1 dead, 5 days; 1 survived	—
1	2 survived 5 days	2 died, 2 days
1/3	2 survived	2 died, 4 days
1/10	2 survived	2 died, 4 days
1/30	2 survived	1 dead, 5 days; 1 survived
1/60	2 survived	2 survived 5 days

Experiments with Human Urine

When acid-hydrolyzed casein was replaced by human urine as dialysate, results similar to those described above were obtained. Once again the toxin was detoxified and at the same time became colored. There was no change in Lf titer but the Kf became prolonged.

Attempts were then made to isolate the active substance or substances from urine. The pH was adjusted to 3.5–4.0 and the urine partially frozen. The precipitate, known as brick-dust deposit, and supernatant were tested separately and both contained substances which reacted with toxin when oxidized by H_2O_2 in the presence of myeloperoxidase. The active material in the supernatant could be absorbed by charcoal and eluted by acetone,

methanol, or ethanol. At least two different active substances appear to be present. They have been purified and separated from one another by absorption and countercurrent distribution experiments. Neither substance has been isolated, as yet, in a sufficiently pure state for definite characterization.

The precipitate which forms when urine is adjusted to pH 3.5–4.0 and cooled, has a high uric acid content. Accordingly uric uric acid was tested in order to find out whether it could act as a cofactor in the peroxidative detoxification of diphtheria toxin.

Experiments with Uric Acid

When a mixture of purified diphtheria toxin and myeloperoxidase is dialyzed against a dilute solution of recrystallized uric acid (0.0005 M) and hydrogen peroxide (0.00025 M), there is a prolongation of the flocculation time although the

TABLE III

Peroxidation of Diphtheria Toxin in the Presence of Uric Acid

20 ml. diphtheria toxin (fraction 4) containing 200 Lf/ml. and 10 μg. myeloperoxidase per ml. was dialyzed against 400 ml. of 0.0005 M uric acid and 0.00025 M H_2O_2 in phosphate buffer pH 7.1.

Duration of dialysis	Combining power	Kf_{10}
hrs.	*Lf/ml.*	*min.*
0	200	9
12	200	65
24	200	95
48	190	115

Lf titer remains constant. No change in color was observed. A typical experiment is summarized in Table III. When this modified toxin was injected into guinea pigs, the results were quite different from those obtained after peroxidative detoxification in the presence of hydrolyzed casein or the other above mentioned cofactors from the urine. As seen from Table IV, even the equivalent of 30 M.L.D. of uric acid–modified toxin failed to kill the animals within 5 days. However, all the animals receiving uric acid–modified toxin in doses corresponding to between 1 and 30 M.L.D. developed paralysis within 9 to 24 days. The experiment described in Table IV was repeated three times, always with the same result.

Guinea pigs injected with toxin treated with uric acid and hydrogen peroxide in the presence of myeloperoxidase generally began to show symptoms of paralysis within 2 to 3 weeks. At first the hind limbs became weak and the animals showed difficulty in moving about. Later more muscle groups became involved and death finally resulted, apparently from respiratory paralysis. These symptoms were never observed in guinea pigs injected with toxin de-

toxified in the presence of hydrolyzed casein or the acetone-soluble substances from urine. Postmortem examinations[5] of animals which died with paralysis failed to reveal significant pathological lesions in the brain, spinal cord, nerve fibres, heart muscles, liver, spleen, or adrenal glands.

Peroxidative modification of diphtheria toxin of the type just discussed appears to be quite specific for uric acid. No detoxification occurs when uric acid is replaced by xanthine, hypoxanthine, allantoin, or alloxan. No change in Kf_{50} value or detoxification occurs when the toxin and myeloperoxidase

TABLE IV

Toxicity of Diphtheria Toxin after Peroxidation in the Presence of Uric Acid

20 ml. purified diphtheria toxin (fraction 4) containing 200 Lf/ml. and 10 μg. myeloperoxidase per ml. was dialyzed against 400 ml. of 0.0005 M uric acid and 0.00025 M H_2O_2 in phosphate buffer at pH 7.1. After 24 and 48 hours, dialysis samples were tested by subcutaneous injection into 240 to 270 gm. guinea pigs.

Amount of treated diphtheria toxin injected	Toxicity	
	After 24 hrs.' dialysis	After 48 hrs.' dialysis
Lf units		
3	Died, 5 days	Died, 3 days
1	Died, 3 days	Paralysis, 12 days
1/3	Paralysis, 9 days	Paralysis, 24 days
1/10	Paralysis, 15 days	Paralysis, 23 days
1/30	Paralysis, 17 days	No symptoms
1/60	No symptoms	No symptoms

are dialyzed against uric acid in the absence of hydrogen peroxide nor when toxin alone is dialyzed against uric acid and hydrogen peroxide.

DISCUSSION

The experiments which have been described demonstrate clearly that purified diphtheria toxin is detoxified by low concentrations of hydrogen peroxide and myeloperoxidase or horseradish peroxidase *only* in the presence of certain oxidizable, dialyzable cofactors. Such cofactors appear to be widely distributed and are present not only in crude culture media but have been found in acid-hydrolyzed casein and in urine. Urine appears to contain a number of distinct substances, one of which has been identified as uric acid, which can modify diphtheria toxin in the presence of hydrogen peroxide and peroxidases. Fractions containing the active cofactors, both from casein hydrolysate and from urine, are themselves oxidized by H_2O_2 and peroxidase in the absence of diphtheria toxin or other protein. Detoxification occurs only if the oxidation

[5] We are grateful to Dr. Murray Angevine, Department of Pathology, University of Wisconsin, for these examinations.

actually takes place in the presence of the toxin. It thus seems probable that the detoxification mechanism depends on the reaction of some intermediate oxidation product of the cofactor with certain groups on the toxin molecule. Studies on the peroxidative oxidation of uric acid will be described in a separate paper (10).

Toxin modified by the action of hydrogen peroxide and cofactor in the presence of peroxidase behaves as a toxoid, since although 99 per cent of the original toxicity is lost it is still capable of flocculating to titer with antitoxin, though with an increased flocculation time.

Highly purified diphtheria toxin containing close to 2200 Lf/mg. nitrogen was isolated by Eaton (11, 12) and by Pappenheimer (8). In the present study, we have isolated toxin of the same degree of purity in reasonably good yield by a relatively simple procedure. The most active material (fraction 4, Table I) was precipitated between 55 and 65 per cent saturation with ammonium sulfate and showed a high degree of homogeneity on electrophoresis. Fractions precipitated below 55 per cent saturation with ammonium sulfate flocculated more slowly with antitoxin, contained fewer Lf units per milligram nitrogen and fewer M.L.D. per Lf unit, and contained a high proportion of material with a different mobility from that of fraction 4. The prolonged Kf and lower toxicity of fractions precipitated by less than 55 per cent saturation with ammonium sulfate (see also references 8 and 12) suggest that either the toxin in the original culture filtrate was not present as a homogeneous protein or it became modified during fractionation.

It has been known for many years that freshly harvested diphtheria toxin and other toxins undergo spontaneous detoxification on standing, even when stored in the cold. This loss in toxicity is *not* accompanied by any change in immunizing power or capacity to react with antitoxin. Ehrlich (13) gave the name toxoid to toxin modified in this way. It has been noted by others who have attempted to purify bacterial toxins that fractionation may remove antigenic material of low toxicity. Thus Pappenheimer (8, 14) noted that diphtheria toxin precipitated at low ammonium sulfate concentration flocculated very slowly and possessed low toxicity per Lf unit; the crystalline tetanus toxin isolated by Pillemer *et al.* (15) was twice as toxic per Lf unit as was the original culture filtrate; Abrams *et al.* (16) noted an increase in toxicity per antigenic unit during fractionation of *botulinus* toxin. Our results indicate that the yield of purified diphtheria toxin of high toxicity with properties similar to those of fraction 4 is considerably greater with fresh toxin than with toxin that has been stored for some time before fractionation.

It seems likely that the well known spontaneous detoxification which occurs on aging diphtheria toxin may be brought about by a peroxidative process similar to that described in these experiments. It is known that the diphtheria bacillus produces peroxidase and other hemin derivatives with per-

oxidative activity. We have shown that the required oxidizable cofactors are present in crude culture filtrates and it is probable that hydrogen peroxide will be formed in low concentration through slow auto-oxidation of reducing substances present in such filtrates. It has been observed that purified toxin does not lose its toxicity when stored over long periods of time and mere dialysis of fresh culture filtrates appears to increase the stability of the toxin markedly. Finally, it may be significant that it is far more difficult to remove the color from an aged toxin by fractionation than from a freshly harvested culture filtrate. So far as we are aware, the only other hypothesis suggested to explain spontaneous toxoid formation is that advanced by Pillemer and Robbins (17) in the case of tetanus toxin. Pillemer has observed that crystalline tetanus toxin loses toxicity on standing at 0°C. This change is accompanied by an increase in sedimentation constant from 4.5 S to 7 S and suggests that in this case, toxoid formation is associated with the formation of an atoxic dimer (18). An analogous change has not been observed in the case of diphtheria toxin.

The experiments with uric acid are of interest since the toxin modified by peroxidation, in this case, produces late paralysis over a wide range of concentration. It has always been difficult to produce diphtheritic paralysis uniformly in animals. Ehrlich (13) observed paralysis in guinea pigs injected with incompletely neutralized mixtures of toxin and antitoxin. Later workers have also observed paralysis generally after repeated small sublethal doses of toxin. Feiner (19) has reported a high percentage of paralyses in guinea pigs injected intravenously with a single dose (0.1–0.4 M.L.D.) of highly purified diphtheria toxin. Animals injected subcutaneously did not develop paralysis to the same extent. While the number of animals used in the present experiments is admittedly small, the results suggest that toxin can be modified by uric acid and H_2O_2 in the presence of peroxidase so that late paralysis occurs in animals injected with amounts corresponding to between 1 and 30 M.L.D. of the original toxin.

SUMMARY

The mechanism of the peroxidative oxidation of diphtheria toxin has been investigated. It has been found that this reaction requires the presence of an oxidizable, dialyzable cofactor in addition to hydrogen peroxide and peroxidase. Cofactors are present in a variety of materials and have been partially purified from urine.

The flocculation titer of toxin modified by peroxidation remains unchanged even after 99 per cent or more of the toxicity has been destroyed. The change thus appears analogous to the conversion of toxin to toxoid in the presence of formalin.

It is suggested that the well known conversion of crude diphtheria toxin to toxoid which occurs on storage or aging may be due to a slow peroxidative process.

BIBLIOGRAPHY

1. Agner, K., *Acta physiol. Scand.*, 1941 **2,** suppl. 8.
2. Theorell, H., and Akeson, A., *Ark. Kem., Mineral., och Geol.*, 1943, **17B,** No. 7.
3. Theorell, H., and Paul, K. G., *Ark. Kem., Mineral., och Geol.*, 1944, **18A,** No. 12.
4. Agner, K., *Nature*, 1947, **159,** 271.
5. Agner, K., *Fed. Proc.*, 1948, **7,** 140.
6. Agner, K., *Fed. Proc.*, 1949, **8,** 178.
7. Mueller, J. H., and Miller, P. A., *J. Immunol.*, 1941, **40,** 21.
8. Pappenheimer, A. M., Jr., *J. Biol. Chem.*, 1937, **120,** 543.
9. Theorell, H., *Ark. Kem., Mineral., och Geol.*, 1942, **16A,** No. 2.
10. Agner, K., Data to be published.
11. Eaton, M. D., *J. Bact.*, 1936, **31,** 347.
12. Eaton, M. D., *J. Bact.*, 1936, **31,** 367.
13. Ehrlich, P., Studies in Immunity, New York, J. Wiley and Sons, 1910.
14. Pappenheimer, A. M., Jr., *J. Bact.*, 1942, **43,** 273.
15. Pillemer, L., Wetter, R. G., and Grossberg, D. B., *Science*, 1946, **103,** 615.
16. Abrams, A., Kegeles, G., and Hottle, G. A., *J. Biol. Chem.*, 1946, **164,** 63.
17. Pillemer, L., and Robbins, K. C., *Ann. Rev. Microbiol.*, 1949, **3,** 265.
18. Pillemer, L., and Moore, D. H., *J. Biol. Chem.*, 1948, **173,** 427.
19. Feiner, R. R., *J. Immunol.*, 1941, **42,** 273.