

THE INACTIVATION OF BIOLOGICALLY ACTIVE PROTEINS, AND
THE VIRUS OF WESTERN EQUINE ENCEPHALOMYELITIS
BY PERIODIC ACID

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When the toxic somatic antigens of *Shigella paradysenteriae* are treated with periodic acid a rapid loss of serological activity occurs accompanied by a marked diminution in the toxicity of the lipocarbohydrate-protein complex (1). The chemical reaction is probably not confined to an oxidation of the carbohydrate moiety of the antigen, and may well involve the protein component, for there is reason to believe that the toxin is an integral part of the latter and is not associated with the carbohydrate constituent (2).

Beyond the fact that periodic acid will oxidize certain amino acids (3), little is known of its action upon native proteins. Nor is it known whether proteins endowed with biologically active properties can be rendered inactive by the reagent under physiological conditions of pH. Recently it was pointed out that the virus receptor group of fowl erythrocytes is destroyed by periodate, and hence might be carbohydrate in nature (4). That this deduction may not be valid, however, will be seen from the following, for it will be shown that certain well defined proteins undergo radical chemical and biological changes when subjected to the action of the reagent. These changes are such that they destroy not only the biological activity of the protein, but in the one instance studied this change was accompanied by a profound alteration in absorption spectrum. In addition, it will be demonstrated that the virus of Western equine encephalomyelitis which, for lack of better evidence, has come to be regarded as protein in nature (5) is rapidly and completely inactivated by the action of the reagent in neutral solution.

THE ACTION OF PERIODIC ACID ON PROTEINS

Materials and Methods.—The crystalline ribonuclease used in these studies was kindly supplied by Dr. M. McCarty. The activity of the enzyme before and after treatment with periodic acid was determined by the procedure of Kunitz (6).

The Type III pneumococcus antibody globulin was prepared by the dissociation of an immune precipitate as described later. Total precipitable nitrogen was estimated by micro-Kjeldahl. The decrease in activity of the immune globulin on treatment with lithium periodate was determined photometrically using the homologous specific polysaccharide as

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precipitinogen (7). 0.1 M lithium periodate in 0.02 M lithium phosphate was prepared by dissolving 2.28 gm. $\text{HIO}_4 \cdot 2\text{H}_2\text{O}$, and 10 ml. of 0.2 M H_3PO_4 in 50 ml. H_2O followed by the addition of 20 ml. of 1.0 N LiOH and diluting to 100 ml. in a volumetric flask.

Immune sera to the native and inactivated immune globulins were obtained from two groups of rabbits which had received intravenous injections of the two proteins. 4.0 mg. of the respective proteins were injected daily for 6 days, followed by an 8 day rest period. The course of injections was repeated, and the sera collected 8 days after the last injection.

The Inactivation of Ribonuclease by Periodic Acid

In order to study the effect of periodic acid on crystalline ribonuclease, a solution of the latter was subjected to action of periodate at pH 5.1 and at

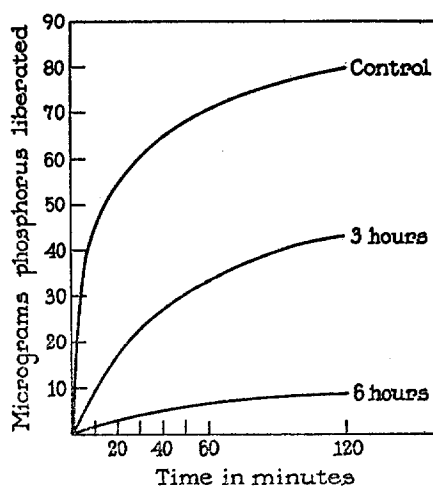


FIG. 1. Liberation of soluble phosphorus from ribonucleic acid by ribonuclease before and after treatment with periodic acid.

25°C. for varying intervals of time. The activity of the enzyme solution was determined and compared with that of untreated enzyme.

A stock solution of ribonucleic acid containing 0.5 mg. phosphorus per ml. in 0.1 M acetate buffer was prepared. A solution of ribonuclease containing 0.10 mg. protein nitrogen per ml. was likewise prepared in 0.01 M acetate buffer and stored at 0°C. The periodic acid-buffer solution was made by mixing equal parts of 0.2 M periodic acid and 0.75 M sodium acetate. The pH of the solution was 5.1.

To study the effect of periodic acid on the enzyme, 5.0 ml. of the latter was mixed with 5.0 ml. of reagent at 25°C. and the mixture allowed to stand. At the end of 3, 6, and 24 hours 2.0 ml. of solution was removed, transferred to a cellophane bag containing 0.3 ml. of 50 per cent glucose, and dialyzed in a rocking device for 4 hours against a flow of distilled water. The contents of the bag were transferred quantitatively to a 10 ml. volumetric flask, and the activity of the enzyme determined. 5.0 ml. of enzyme, now containing 0.01 mg. protein nitrogen per ml., was mixed with 5.0 ml. of stock ribonucleic acid solution, and the liberated phosphorus determined at the end of 30, 60, and 120 minutes. The results are presented graphically in Fig. 1. For purposes of comparison this figure includes the activity curve of the untreated ribonuclease.

From the results presented in Fig. 1 it is apparent that treatment of the enzyme at pH 5.1 with periodic acid brings about a gradual loss of biological activity, and this change is not rapid. Inactivation is apparently complete after contact with the reagent for 24 hours, for it is not possible to demonstrate the liberation of phosphorus from ribonucleic acid by the enzyme treated for this period of time. The nature of the chemical change in the enzyme molecule brought about by the oxidant is not known, but that it is profound is evident from the fact that a complete loss of biological activity occurs.

The Inactivation of Type III Pneumococcus Antibody by Periodic Acid

Preparation of Antibody.—A second protein was chosen for study in order to determine whether biological activity of an entirely different nature would be affected by periodic acid. Type III pneumococcus immune horse globulin was selected, because it can be had in relative abundance.

The purified antibody was prepared by dissociating an immune precipitate (8) obtained from the interaction of 3 liters of antipneumococcus horse serum Type III, and the appropriate quantity of homologous type specific polysaccharide. The serum was diluted to 13 liters with 0.9 per cent NaCl; 0.25 gm. of Type III polysaccharide dissolved in 1 liter of saline solution at 5°C. was added. This quantity sufficed to carry down the major part of the antibody, but still left a slight excess in the supernate.

After 2 hours the immune precipitate was spun at 0°C., washed five times with cold saline solution, and suspended in 400 ml. of 25 per cent ammonium sulfate. The solution was stirred and warmed to 37°C. for an hour, spun, and then diluted to 50 per cent saturation with saturated ammonium sulfate. The dissociated antibody which precipitated was collected by centrifugation, and twice reprecipitated by half saturation with ammonium sulfate. The antibody was then dissolved in water and dialyzed free of SO_4^{--} against 0.9 per cent NaCl. Small amounts of precipitate which separated during the dialysis were centrifuged and discarded. From 25 to 38 per cent of the protein originally present in the immune precipitate was recovered by this procedure. The dissociated antibody prepared as described above varied in its specific precipitable nitrogen content from 65 to 82 per cent.

Inactivation of Pneumococcus Antibody by Periodic Acid.—A solution of Type III antibody containing 1.0 mg. nitrogen per ml. was treated with an equal volume of 0.05 M LiIO_4 at pH 7.2. At varying time intervals 0.5 ml. samples were removed, added to 0.2 ml. 50 per cent glucose in a 5.0 ml. volumetric flask, and diluted to the mark with 0.9 per cent NaCl. Samples of 0.40, 0.20, and 0.10 ml. were removed, placed in calibrated tubes, diluted in each instance to exactly 1.00 ml. Then 1.00 ml. of a dilution of Type III polysaccharide containing 10 micrograms of carbohydrate was added to each tube. After 30 minutes the turbidity was measured in a phototurbidometer, and the result recorded (Fig. 2). When the classical quantitative precipitin reaction was carried out on the various treated antibody solutions, the results were essentially the same as those obtained photometrically. It can be seen that the precipitability of the immune globulin is rapidly impaired by contact with the reagent, and is entirely destroyed when the globulin remains in contact with the periodate for 24 hours (not shown on graph).

Change in the Absorption Spectrum of Immune Globulin Treated with Lithium Periodate at pH 7.2.—A solution of Type III pneumococcus anticarbohydrate-immune globulin containing 20.0 mg. protein per ml. was treated with an equal volume of 0.05 M LiIO_4 at pH 7.2 for 24 hours. The reagent was removed by rapid dialysis against physiological salt solution, and diluted to a concentration of 1.0 mg. protein per ml. in 0.02 M phosphate buffer at pH 7.6. The

absorption spectrum of this material was measured and compared with that of untreated immune globulin of the same concentration in the same buffer. The absorption spectrum is shown in Fig. 3, where *a* represents the absorption spectrum of the native globulin, and *b* that of the globulin after treatment with lithium periodate.

It is evident that a marked change in the absorption spectrum has taken place. Not only is there an increase in absorption at the maximum as well as a slight shift to a shorter wave length, but there is a relatively greater increase in the minimum absorption coefficient. The reason for this is not as yet known, but it should be pointed out that Gates (9) observed a similar shift in the absorption spectrum of crystalline pepsin after irradiation with ultraviolet light. Furthermore, Hicks and Holden (10) have measured the absorption spectra of proteins denatured by alkali and by alcohol, and observed an

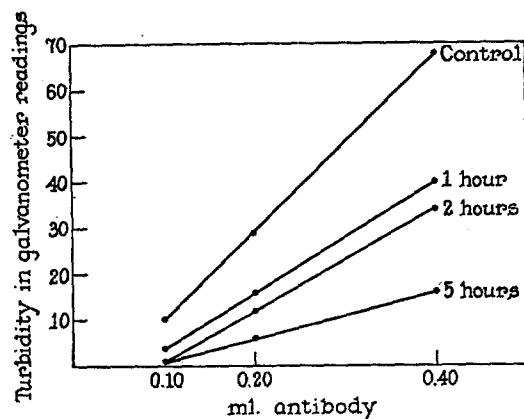


FIG. 2. Inactivation of Type III pneumococcus antibody globulin by lithium periodate.

increased absorption through the entire range of wave lengths. Whether the change in absorption spectrum of the immune globulin which follows treatment with periodate represents true denaturation comparable to that brought about by alcohol or alkali cannot yet be said with certainty. In this connection, however, it should be pointed out that after such treatment, a portion of protein, but by no means all, will precipitate in the presence of 0.9 per cent sodium chloride when the pH of the solution is shifted from neutrality to 4.8.

Specificity of Antisera Prepared by Immunization with Inactivated Immune Globulin.—That a marked chemical change has taken place when immune globulin is treated with periodate at pH 7.2 is apparent from the foregoing experiments. Whether this change is sufficiently great to affect the antigenicity of the altered protein was not known.

A group of rabbits was injected with daily doses of 4 mg. of the active protein for 6 consecutive days, following a rest period of 8 days; the course of injection was repeated, and 8 days following the last injection, the animals were bled. A second group of animals was likewise

immunized with untreated immune globulin. The sera were then tested for homologous precipitins (Table I) and heterologous precipitins (Table II).

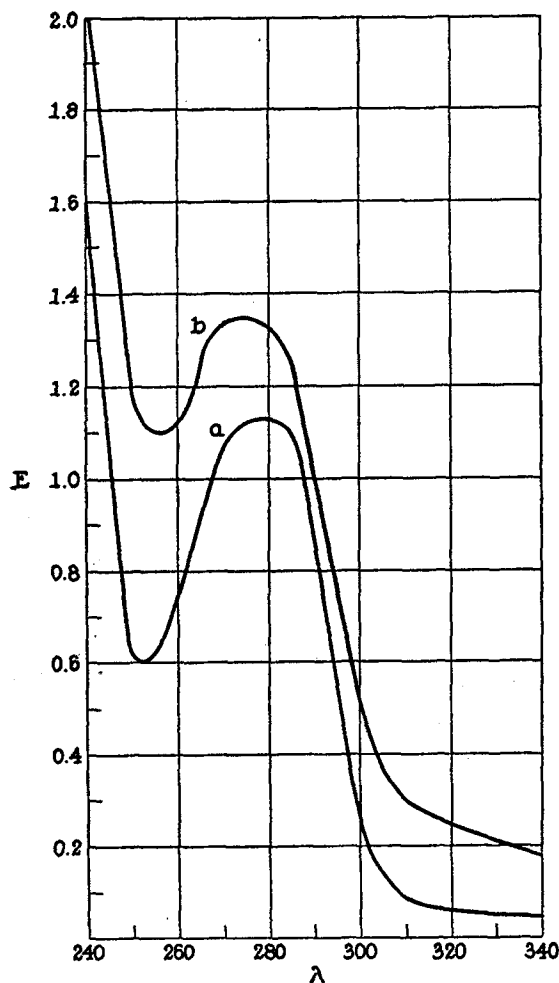


FIG. 3. Absorption spectra of Type III pneumococcus antibody globulin before and after treatment with lithium periodate.

Immune globulin, treated with lithium periodate for a period of 24 hours and completely inactivated, is fully antigenic, as can be seen from Table I. Furthermore, when the inactivated protein is tested in the antiserum prepared by injecting rabbits with untreated and fully active immune globulin, marked precipitation occurs (Table II). If the respective sera are absorbed with the heterologous antigen, and then tested with the homologous protein, no reaction occurs indicating that the antibody is completely removed (Table III) by the

heterologous protein. From the results of these experiments it can be concluded that although destruction of biological activity takes place when the

TABLE I
Homologous Precipitin Reactions of Sera of Rabbits Immunized with Type III Pneumococcus Antibody Globulin, before and after Treatment with Lithium Periodate

Rabbit No.	Immunized with*	Test antigen	Final dilution of test antigen			
			1:10,000	1:50,000	1:100,000	1:200,000
1	G _{III}	G _{III}	+++	++	+±	+
2			++++	+++±	+±	+
3	G _{III} (HIO ₄)	G _{III} (HIO ₄)	+++±	++±	++	+
4			+++	++	+±	+

* In Tables I and II, G_{III} refers to untreated Type III pneumococcus immune globulin; G_{III} (HIO₄) to the globulin treated 24 hours with LiIO₄.

TABLE II
Heterologous Precipitin Reactions of Sera of Rabbits Immunized with Type III Pneumococcus Antibody Globulin, before and after Treatment with Lithium Periodate

Rabbit No.	Immunized with	Test antigen	Final dilution of test antigen			
			1:10,000	1:50,000	1:100,000	1:200,000
2	G _{III}	G _{III} (HIO ₄)	+++±	++±	+±	+
3	G _{III} (HIO ₄)	G _{III}	+++	++	+	±

TABLE III
Cross-Precipitin Reactions in Absorbed Rabbit Sera

Serum of rabbit immunized with:	Serum absorbed with	Test antigen used	Final dilution of test antigen			
			1:10,000	1:50,000	1:100,000	1:200,000
G _{III}	Unabsorbed	G _{III}	+++±	++±	+±	+
	G _{III} (HIO ₄)	"	0	0	0	0
	"	G _{III} (HIO ₄)	0	0	0	0
G _{III} (HIO ₄)	Unabsorbed	G _{III} (HIO ₄)	+++	++	+	±
	G _{III}	"	0	0	0	0
	"	G _{III}	0	0	0	0

immune globulin is treated with periodate, neither the capacity of the protein to incite specific immune bodies is destroyed, nor is the species specificity of the protein lost. If treatment of the immune globulin with periodate is accompanied by denaturation, it is difficult to understand why the specificity of the antibody elicited by the treated protein is not altered. The consensus of

opinion is that the denaturation of proteins endows them with a new and changed specificity (11).

THE ACTION OF PERIODIC ACID ON A VIRUS

The action of potassium and lithium periodate on the virus of Western equine encephalomyelitis was then investigated.

Materials and Methods.—The Rockefeller Institute strain of the virus used in these studies was stored frozen, at about -70°C ., in the form of 20 per cent mouse brain in 50 per cent normal rabbit serum. The LD_{50} of the thawed material as tested in the Rockefeller Institute strain of albino mice, was about $10^{-8.5}$. Before determining the effect of periodate upon the virus, the latter was first diluted with an equal volume of 10 per cent normal rabbit serum, the suspension was then centrifuged at 10,000 R.P.M. for 30 minutes, and the supernate, containing the virus, was employed. To 1 ml. of the supernate virus were added 2 ml. of 0.01 M phosphate buffer at pH 7.0, 1 ml. of 0.05 M KIO_4 solution, and 5.5 ml. of 0.4 per cent NaCl solution. After a period of contact varying from 7 minutes to 3 hours at 37°C . (depending on the particular experiment of those to be described later), 0.5 ml. of 50 per cent dextrose solution was added to decompose the periodate. Thus an additional tenfold dilution of virus was obtained. The mixture was then dialyzed under sterile conditions in a mechanical rocker for 4 hours against a constant flow of 0.01 M phosphate buffer at pH 7.0. The materials were removed and titrated for virus activity by the intracerebral inoculation of mice, making dilutions in 10 per cent normal rabbit serum (12). For each experiment the appropriate controls were included as well as a titration of the same untreated virus sample as employed in the test (Table IV). In later tests, the lithium salt of periodic acid, because of greater solubility, was substituted for the corresponding potassium salt. When the lithium salt was employed certain other modifications were introduced; e.g., 1 ml. of 20 per cent virus was added to 1 ml. of 0.05 M or 0.1 M LiIO_4 in 0.02 M lithium phosphate buffer at pH 7.2. After contact for varying periods of time at 37°C ., 0.2 ml. of 50 per cent dextrose solution was added. The procedure of dialysis, dilution of dialyzed materials, and mouse test was otherwise unchanged. The complement-fixation tests as here reported were performed after the manner described by Casals (13). The virus exposed to the periodate for 10, 30, and 60 minutes comprised the antigen. The latter was serially diluted up to 1/64; the serum from 0 to 1/128. Known standard equine virus antisera and those against St. Louis and Japanese B encephalitis viruses were tested as controls along with the serum from mice immunized as described in the next paragraph (13).

To test for the presence of an immunity developed to the periodate-treated virus, mice were injected intraperitoneally with material inactivated for 10, 30, and 60 minutes; 120 to 210 mice were included in each test. Six immunizing doses of 0.25 ml. each were given, in two series of three consecutive daily injections with a 5 day interval between the series. To test for any immunity that might have been developed, 11 days after the last dose, dilutions of 10^{-3} , 10^{-5} , 10^{-6} , and 10^{-7} of virus were inoculated intracerebrally into 6 or more mice for each dilution. Similar injections were given to uninoculated animals.

For the detection of complement-fixation antibody, serum from these mice was collected. For determination of virus-neutralization antibody, serum was obtained from rabbits which had received repeated doses of 3 to 10 ml. of virus intraperitoneally over a period of 29 days. The virus employed was a sample which had been in contact with the periodate for 10 and for 30 minutes. The neutralization and complement-fixation tests for these antibodies were performed in the manner already described (12, 13).

Effect of Periodate on Virus Titer.—A prompt and marked inactivation of

the virus of Western equine encephalitis occurred after exposure to potassium or lithium periodate. Table IV, which represents but a single test, shows that while some inactivation of virus was demonstrable within 10 minutes contact with a 0.05 M solution of the reagent more than 25 million doses were inactivated after a period of 2 hours. These observations were confirmed by the results of other tests in which it was found that within 7 to 10 minutes from 100,000 to 1,000,000 doses of virus were rendered inactive by periodate of 0.1 M concentration. To summarize the results of a number of experiments, it would appear that both 0.05 M potassium and 0.05 or 0.1 M lithium periodate could inactivate an appreciable amount of virus within a few minutes. The effect, however, increased with the duration of the time of contact so that most of the

TABLE IV
Showing Inhibition of the Virus of Western Equine Encephalitis by 0.05 M LiIO_4 , and the Influence of Time of Contact upon the Reaction

Virus dilution	Tests				Controls		
	Time of contact with LiIO_4 at 37°C.				Virus dilution	1 hr.	2 hrs.
	10 min.	30 min.	1 hr.	2 hrs.			
10^{-1}	5/5*	5/5	3/5	2/5	10^{-7}	5/5	5/5
10^{-2}	5/5	3/5	1/5	0/5	10^{-8}	5/5	4/5
10^{-3}	5/5	2/5	0/5	0/5	10^{-9}	1/5	0/5
10^{-4}	3/5	0/5	0/5	0/5	10^{-10}	0/5	0/5
LD_{50}	$\approx 4.2\ddagger$	2.5	1.3	<1.0		8.6	8.4

* Number of mice succumbed to virus infection over number of mice used.

† Reciprocal of log of dilution.

virus added was made ineffective within $\frac{1}{2}$ to 1 hour, and all the virus that could possibly be used within the limits of the tests, *i.e.* over 300 million lethal doses, was rendered inert within 2, $2\frac{1}{2}$, or 3 hours.

The reduction in titer of the virus by means of periodate was a function not only of time of contact (Table IV) but also of temperature. Thus, when the test was carried out at 37°C., as compared with 4°C., a greater degree of inactivation ensued at the higher temperature, greater by at least 20 per cent. It was also found that the pH of the mixtures, which is varied but little before and after dialysis, was not a factor in the reduction of the titer of the virus. For example, electrometric determinations revealed that the pH of the mixtures usually ranged from 6.8 to 7.1, either at the time of mixing or before their inoculation into animals.

Although the data are not given it was shown that the periodate ion rather than potassium or lithium ion was responsible for the inactivation of the virus. Also, the reduction in titer of the virus was the result of the action of the

periodate itself, not that of a secondary substance which might have developed from the interaction of the component materials used in the test.

Effect of Periodate on the Antigenicity of the Virus.—The problem of the effect which periodate exerted on the antigenicity of the virus was studied from two angles. In the first tests, attempts were made to immunize mice to a test dose of virus by injecting periodate-treated virus repeatedly. In the second series of experiments the serum of such treated animals was examined for the presence of induced complement-fixing antibody. In addition, a search was undertaken for virus-neutralizing antibody in the sera of rabbits injected repeatedly with periodate-treated virus.

In summary, the treated virus failed in all instances to induce either active or passive immunity, even when exposure to the reagent was as short a time as 10 minutes. Such material also did not bring about the development of serum antibody, either complement-fixing or virus-neutralizing. To be sure, virus treated with the periodate for 10 or 30 minutes and used in these tests as antigen, contained a certain amount of active virus and a degree of immune response was at times noted. The response, however, was no greater than that expected from the immunogenic action of the small amount of residual virus present in the inocula. The relationship of the amount of virus used to the degree of immunity induced has already been established (14, 15). To conclude, just as the virus of Western equine encephalitis was inactivated rapidly by exposure to the action of periodate, so was its antigenicity completely destroyed, even within as short a time as 10 minutes, under the experimental conditions here employed.

DISCUSSION

Periodic acid is an oxidizing agent which has been extensively employed in the study of the structure of carbohydrate and steroid derivatives. Its value can be attributed to the fact that in general its oxidizing action is selective for it severs linkages only between those carbon atoms bearing adjacent hydroxyl, or hydroxyl and amino groups. We became interested in the reagent when it was observed that the somatic antigens of dysentery bacilli were readily detoxified by periodate. Since the toxic component of these microorganisms appears to be intimately associated with the protein and not the carbohydrate portion of the antigenic complex, it was reasonable to assume that the former must be altered chemically by the reagent. A perusal of the literature revealed little concerning the action of periodate upon proteins. Desnuelle and Antonin (16) observed that when several proteins, including egg albumin, were treated with sodium periodate there was no liberation of formaldehyde and assumed that the reagent induced only an insignificant chemical change in these proteins. That certain amino acids, such as serine and threonine, are quantitatively oxidized is of course well known.

In the present investigation it has been demonstrated that when a subtle biological function is involved, this latter may be readily impaired when the protein concerned is subjected to the action of periodate at physiological values of hydrogen ion concentration. Not only was the biological activity of two widely divergent proteins, an enzyme and an antibody, lost, but the pathogenicity of a virus was destroyed. It is of interest that although the serological specificity of Type III pneumococcus immune globulin was rapidly impaired, and finally destroyed by periodate, the capacity of treated immune globulin to elicit antibody in experimental animals was not. This is in direct contrast to the results observed with Western equine encephalomyelitis virus, where contact with the reagent, even for a very brief time interval destroyed, under the experimental procedures used, its infectivity and its ability to elicit specific antibody.

The nature of the chemical action of periodic acid upon the proteins studied cannot at the present time be elucidated. No intensive attempts have been made as yet to disclose the mechanism of the action of the reagent upon these substances beyond the demonstration that a change in the absorption spectrum of immune globulin accompanies contact with the reagent. Whether certain terminal amino acids such as serine or threonine are oxidized cannot yet be said. That certain proteins are readily inactivated by the reagent is evident from the foregoing experimental data. One should be reserved in the assumption that because the biological function of an unknown substance is impaired by contact with periodate, the material should be classified as a carbohydrate.

SUMMARY

The action of periodic acid on two biologically active proteins, crystalline ribonuclease and pneumococcus Type III immune globulin, and on the virus of Western equine encephalomyelitis has been studied. The biological activity of the two proteins and the pathogenic action of the virus were destroyed by the reagent; the specific antigenicity of the immune globulin was retained, however, but that of the equine virus was lost. The bearing of these reactions on the chemical alteration of the respective substances has been discussed.

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