Experimental

Materials.—The preparations of botulinum toxin, type A, used in this work were isolated by either the method of Abrams, *et al.*,[3] or by a method based on differential ultracentrifugation.[7] Only crystalline preparations homogeneous in the ultracentrifuge at *p*H 3.8, with a specific activity, when isolated, of at least 200×10^6 LD_{50} per mg. N, were used. The toxin was stored in acetate buffer at 5° and adjusted immediately before use with an appropriate phosphate buffer.

The ketene was produced by the pyrolysis of acetone in an apparatus similar to that designed by Herriott.[12] The rate of production was measured by the neutralization of standard alkali under conditions of the actual acetylations.

Toxicity.—The number of LD_{50} per ml. was determined by injecting 16–20 gram mice intraperitoneally with 0.5 ml. of toxin diluted with a sterile 1% disodium phosphate solution containing 0.2% gelatin and adjusted to *p*H 6.8. Six mice were used in each group and deaths were recorded for 96 hr. The per cent. kill was plotted against the dose on probit-log dose paper with the probit for 100% killed taken as the probit for five out of six killed plus $^1/_2$ probit unit and the probit of 0% killed taken as the probit of one out of six killed minus $^1/_2$ probit unit.[13] The best straight line was fitted by inspection and the dose corresponding to probit 5 was read off the graph.

Amino Nitrogen.—The extent of N-acetylation was ascertained by the nitrous acid amino nitrogen method of Van Slyke.[14] The reaction was carried out at 30–32° for 20 minutes in a reaction mixture containing 5 ml. of sample, 2 ml. of glacial acetic acid–saturated sodium acetate (1:1) and 2 ml. of sodium nitrite (saturated at 5°). The use of a buffered reaction, first suggested by Rutherford, *et al.*,[15] serves to decrease the volume of gas evolved and the magnitude of the blank analysis.

Phenolic Hydroxyl.—The extent of tyrosine O-acetylation was determined by Herriott's modification of the Folin phenol method at *p*H 8. The solutions were read at 750 mμ in a Beckman model DU spectrophotometer. The standard curve was made with tryptophan.

Nitrogen.—Nitrogen in protein samples was determined by micro-Kjeldahl using a mercuric oxide catalyst and a 4-hr. digestion period.

Flocculation.—One Lf unit represents the amount of toxin which gives the most rapid flocculation with one standard unit of antitoxin.[16] The Kf is the time interval for the earliest flocculation. The determination was carried out at 40° in a water-bath with 1 ml. of diluted toxin and volumes of antitoxin (100 units per ml.) varying from 0.10 to 0.41 ml. using a dilution factor of 1.25.

The Rate of Inactivation of Toxin by Ketene.—Samples of 0.25 ml. of toxin dissolved in 0.5 *M* potassium phosphate buffer at *p*H 6.6 were placed in a 2 cm. diameter vial. The outlet tube of the ketene generator was placed about $^1/_2$ cm. above the surface of the liquid and the sample was swirled slowly. After exposure for a predetermined period, the reaction was stopped by adding 4.75 ml. of gelatin–phosphate diluent. Under these conditions, the absorption rate of ketene into standard alkali was found to be 0.36 mmole per minute.

The Reduction in Amino Nitrogen Due to Ketene Treatment of the Toxin.—Twelve-ml. samples of toxin dissolved in 0.5 *M* potassium phosphate buffer at *p*H 6.4 were placed in 30-ml. beakers and exposed to ketene. The solutions were stirred slowly with a magnetic stirrer. Samples were withdrawn for toxicity tests immediately after exposure and amino nitrogen was determined in duplicate analyses the same day.

The Reduction in Phenolic Hydroxyl Due to Ketene Treatment of the Toxin.—Four-ml. samples of toxin dissolved in 0.5 *M* phosphate buffer at *p*H 6.5 were placed in 30-ml. beakers and exposed to ketene. The samples were swirled during exposure. Samples were withdrawn for toxicity tests and phenol determinations immediately after exposure.

The Effect of Sulfhydryl Reagents on the Activity of the Toxin.—Samples of 0.1 ml. of toxin and varying small volumes (0.025 to 0.20 ml.) of dilute solutions (1×10^{-3} to 1×10^{-5} *M*) of the reagent to be tested, usually *p*-chloromercuribenzoic acid, were added to enough 0.1 *M* phosphate buffer at *p*H 7.0 to make a final volume of 2.0 ml. The mixtures had a *p*H of 6.8–6.9. The reaction was allowed to proceed for 15 minutes at room temperature when samples were withdrawn for toxicity determinations.

The Antigenicity of Ketene Treated Botulinum Toxin.—A 2.5-ml. sample of toxin dissolved in 0.5 *M* phosphate buffer at *p*H 6.5 was exposed to ketene for two minutes. The *p*H was then adjusted back to *p*H 6.5 with 0.03 ml. of 10 *N* NaOH. Four exposures were made on the same sample with *p*H adjustment after each. The final solution had less than 40 LD_{50} per ml. compared to an original activity of 35×10^6 LD_{50} per ml. The solution was diluted to a concentration of 1×10^6 LD_{50} per ml. of original unitage and an alum precipitated toxoid was prepared according to the method of Nigg, *et al.*[17] The toxoid was tested by the constant toxoid method of Hottle, *et al.*,[8] in mice.

Acknowledgment.—The authors are indebted to Mr. W. I. Jones, Jr., for some of the chemical determinations and to Mr. J. T. Duff for carrying out the flocculation tests.

(12) R. M. Herriott, *J. Gen. Physiol.*, **18**, 69 (1934).
(13) E. S. Weiss, *Am. J. Public Health*, **38**, 22 (1948).
(14) D. D. Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929).
(15) *Cf.* footnote *b*, Table I.
(16) Globulin modified antitoxin, Lederle.
(17) C. Nigg, G. A. Hottle, L. L. Coriell, A. S. Rosenwald and G. W. Beveridge, *J. Immunol.*, **55**, 245 (1947).

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[CONTRIBUTION FROM THE CHEMICAL CORPS, FORT DETRICK]

The Reaction of Botulinum Toxin Type A with Nitrous Acid[1]

BY LEONARD SPERO AND EDWARD J. SCHANTZ

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Botulinum toxin, type A, was inactivated rapidly by treatment with nitrous acid in the presence of excess nitrite. This reaction, presumably deamination, followed first-order kinetics. It is shown that the deamination of alanine under these conditions is also first order and that this is to be expected from the rate equation for the reaction. These results provide additional evidence for the essential nature of the free amino groups in the toxicity of this protein.

The reaction of botulinum toxin, type A, with ketene was described in the preceding paper,[2] and it was proposed on the basis of the rate of reaction and analysis of reacting groups that the free amino groups, rather than the phenolic hydroxyl groups, were involved in the inactivation. In order to obtain more definitive information on this point, the reaction of the toxin with nitrous acid has been studied. This reaction, when carried out in the presence of excess nitrite, has been proposed[3] as a means of distinguishing between these two reactive

(1) Presented, in part, before the Division of Biological Chemistry, 128th meeting, American Chemical Society, Minneapolis, Minn., September, 1955.
(2) E. J. Schantz and L. Spero, THIS JOURNAL, **79**, 1623 (1957.)
(3) J. E. Little and M. L. Caldwell, *J. Biol. Chem.*, **147**, 229 (1943).

groups on the following basis: the reaction with tyrosine (either as the free amino acid[4] or in proteins containing essential phenolic hydroxyl groups[5,6]) is slow and of the first order, whereas the deamination of amino groups proceeds much more rapidly and is of the second order.[3] Data are presented in the present paper showing that the inactivation of botulinum toxin with nitrous acid is rapid but follows first-order kinetics in the presence of excess nitrite instead of the predicted second order. It was found also that first-order kinetics are followed in the deamination of a simple amino acid.

Results and Discussion

Data for the inactivation of botulinum toxin by nitrous acid are shown in Table I for two experiments. The conditions of treatment described by Philpot and Small[4] were used, namely, 0.25 *M* acetate buffer at *p*H 4.0 and 0°, except that the nitrite concentration was reduced from 1.0 to 0.33 *M* in order to slow the reaction and obtain more intermediate points. It is apparent that the detoxification proceeded very rapidly and continuously, consistent with the interpretation that the free amino groups of the toxin are required for its toxic activity.

TABLE I

THE EFFECT OF NITROUS ACID[a] ON THE TOXICITY OF BOTULINUM TOXIN

Reaction time, min.	Toxicity, LD_{54}/ml. Expt. 1[b]	Expt. 2[c]
0	4.5×10^7	2.5×10^7
5	4.0×10^7	
10	3.5×10^7	
20	2.8×10^7	1.25×10^7
40	2.0×10^7	8.0×10^6
80	1.0×10^7	3.0×10^6
120		1.2×10^6
160		6.0×10^5
200		2.7×10^5

[a] 0.33 *M* nitrite in 0.25 *M* acetate at *p*H 4.0 and 0°. [b] 1.01 mg. toxin per ml. [c] 0.66 mg. toxin per ml.

The data of Table I are plotted logarithmically against time in Fig. 1. An excellent fit was afforded by the straight line calculated by the method of least squares with correlation coefficients of −0.998 and −0.994. To test the data for a fit to a second-order reaction the reciprocal of the fraction of the original toxicity was plotted against time. A considerable curvature was apparent in this case and least square straight lines gave absurd values for the intercepts on the ordinate. This reaction of the toxin, therefore, should be described as pseudo-unimolecular. The first-order reaction rate constants calculated from the slope of the least-square straight lines are 0.0184 and 0.0226 min.$^{-1}$ for the two experiments, and the respective half-lives are 38 and 31 minutes. This agreement is good considering that it is based on a biological assay and is further evidence for the first-order nature of the inactivation. The starting concentrations of the toxin differ by a factor of more than 1.5, so that if the reaction were second order, the half-life of the toxicity for the second experiment should have been increased to 58 minutes; instead the actual observed value was even less than in the first experiment.

In contrast to the results obtained on botulinum toxin, it has been reported by Little and Caldwell[3] that the reaction of nitrous acid with pancreatic amylase, which also contains essential amino groups, is pseudo-bimolecular in the presence of excess nitrite. To obtain some insight on the nature of these reactions, the deamination of a simple amino acid in the presence of excess nitrite was investigated using alanine as a model. The reaction was followed by stopping it with alkali at predetermined periods and measuring the unreacted alanine by a modified Van Slyke amino nitrogen determination. It was found that the reaction was first order under several conditions of *p*H, alanine concentration and nitrite concentration. A summary of these conditions and the respective first-order reaction rate constants obtained are presented in Table II. The complete course of the reaction for the different *p*H values (runs a, e, f, g) is illustrated in Fig. 2 as a plot of the logarithm of alanine concentration against time. As with the toxin, the least square straight lines gave an excellent fit with the first-order plot of the data. Second-order plots had a pronounced curvature. A comparison of runs b, c and d in Table II demonstrates that the reaction rate constants were substantially unchanged over a threefold range in alanine concentration providing further evidence that the reaction was first order with respect to the amino acid.

TABLE II

THE EFFECT OF *p*H AND THE CONCENTRATION OF ALANINE AND NITRITE ON THE RATE OF DEAMINATION OF ALANINE

Run	*p*H	Concn. of alanine, moles/l.	Concn. of nitrite,[a] moles/l.	First-order reaction const.,[b] min.$^{-1}$ × 10^3
a	4.0	0.011	1.0	27.2
b	4.0	.0056	0.33	4.20
c	4.0	.011	0.33	3.82
d	4.0	.017	0.33	3.57
e	4.5	.017	1.0	7.43
f	3.75	.017	1.0	34.0
g	3.5	.017	1.0	57.7

[a] Buffered with acetate at *p*H 4 and above and with citrate below *p*H 4. [b] From slope of least square straight lines.

The first-order reaction rate constants in Table II actually represent the true reaction rate constant multiplied by a nitrite dependent concentration factor (presumably some function of undissociated nitrous acid). This factor stays essentially constant for any one set of experimental conditions since the nitrite is in excess, but it does produce changes in the apparent first-order constants as the nitrite concentration is changed. This can be done either by a direct change in the total nitrite (*e.g.*, runs a and c) or by a change in the undissociated nitrous acid due to an alteration in *p*H (*e.g.*, runs e and g). No simple expression for this factor has been found which satisfies all of the observations. This is not too surprising in view of the reports in the literature on this type of reaction.

(4) J. St. L. Philpot and P. A. Small, *Biochem. J.*, **32**, 542 (1938).
(5) C. E. Weill and M. L. Caldwell, THIS JOURNAL, **67**, 212 (1945).
(6) I. W. Sizer, *J. Biol. Chem.*, **160**, 547 (1945).

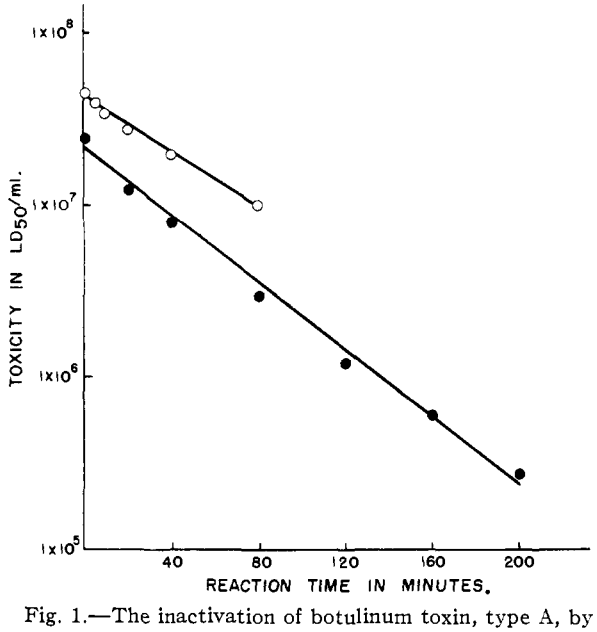

Fig. 1.—The inactivation of botulinum toxin, type A, by nitrous acid. The toxicity data from Table I are plotted logarithmically against time: O, expt. 1; ●, expt. 2.

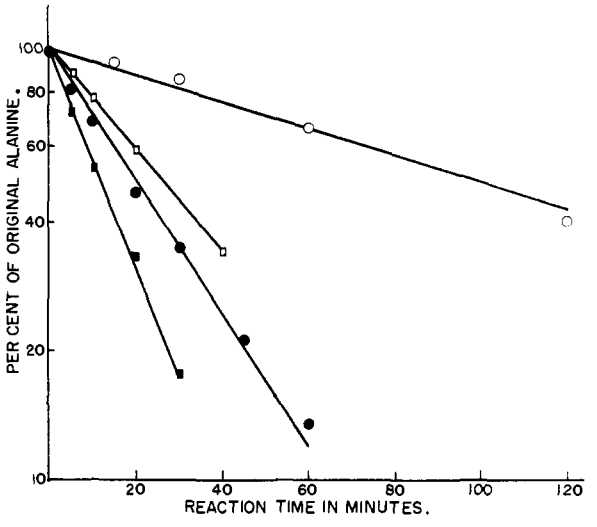

Fig. 2.—The deamination of alanine by nitrous acid at several *p*H values. The per cent. of original alanine is plotted logarithmically against time: O, *p*H 4.5 in acetate, 0.017 *M* alanine (run e); □, *p*H 4.0 in acetate, 0.011 *M* alanine (run a); ●, *p*H 3.75 in citrate, 0.017 *M* alanine (run f); ■, *p*H 3.5 in citrate, 0.017 *M* alanine (run g). The nitrite concentration was 1.0 *M*.

Thus, Taylor[7] found that the reaction of amines, ammonia and amino acids with nitrous acid was trimolecular, with the rate proportional to the first power of the amino compound for all. The kinetics for amino acids showed

$$\text{Rate} \propto (\text{amino acid}^{+-})(HNO_2)^2$$

Dusenbury and Powell[8] found the reaction of ammonia and methylamine with nitrous acid to be bimolecular with the rate proportional to the first power of both ammonium ion and undissociated nitrous acid. Austin, *et al.*,[9] confirmed the results of Taylor but considered that Dusenbury and Powell's work was also valid and attributed the difference to an altered nitrosating agent from the high buffer concentration in the latter's experimental conditions.

The crucial point is that all three groups found the rate proportional to the first power of the concentration of the amino compound. Since under the conditions of deamination which have been employed in the present experiments (a buffered medium with a large excess of nitrite) the concentration of nitrite and undissociated nitrous acid is effectively unchanged throughout the course of the reaction, it follows that, regardless of the function of nitrite or nitrous acid in the rate equation, the equation must reduce to the form of a pseudo-unimolecular reaction. This has been confirmed in our experiments with alanine under a variety of conditions. Further experimental confirmation is furnished in a study by Dunn and Schmidt[10] of the influence of position and temperature on the reaction of aliphatic amino nitrogen with nitrous acid using the volumetric Van Slyke apparatus (in this reaction the nitrite is, of course, in great excess). They believed that the values for K calculated for first-order kinetics agreed sufficiently to establish the fact that the reaction is of the first order.

The first-order kinetics observed for the inactivation of botulinum toxin with nitrous acid in the presence of excess nitrite are, therefore, consistent with the kinetics of deamination of simple amino compounds. Pancreatic amylase is the only other protein with essential amino groups for which the kinetics of nitrous acid inactivation have been determined, and the pseudo-bimolecular reaction found in this case would appear to be atypical on the basis of the present report.

These studies weaken the proposal that the reaction with nitrous acid can be used to distinguish between free amino groups and phenolic hydroxyl groups in protein inactivation. Since both types of inactivation may follow first-order kinetics, the only valid criterion remaining is the rate of the reaction; this, by itself, is insufficient to affix reactivity to a specific group. The reaction does, however, have some merit in this regard for there is apparently a great difference between the rate of deamination and the rate of substitution into the tyrosine ring. Thus under conditions where the half-life of tyrosine in the substitution reaction was 169 minutes,[4,11] alanine was half deaminated in 26 minutes. Further, pepsin and chymotrypsin with essential phenolic hydroxyl groups had half-lives of 160[4] and 420 minutes,[6] respectively, while pancreatic amylase with essential amino groups had a half-life of 14 minutes.[3] Botulinum toxin had

(7) T. W. J. Taylor, *J. Chem. Soc.*, 1897 (1928); 1099 (1928); T. W. J. Taylor and L. S. Price, *ibid.*, 2050 (1929).

(8) J. H. Dusenbury and R. E. Powell, THIS JOURNAL, **73**, 3266 (1951); **73**, 3269 (1951).

(9) A. T. Austin, E. D. Hughes, C. K. Ingold and J. H. Ridd, *ibid.*, **74**, 555 (1952).

(10) M. S. Dunn and C. L. A. Schmidt, *J. Biol. Chem.*, **53**, 401 (1922).

(11) The conditions for the reactions compared herein vary: tyrosine, alanine and pepsin, *p*H 4.0, 1.0 *M*, nitrite; chymotrypsin and pancreatic amylase, *p*H 4.6, 1.0 *M* nitrite; botulinum toxin, *p*H 4.0, 0.33 *M* nitrite.

a half-life of 35 minutes under similar conditions. These data, coupled with the evidence in the preceding paper, indicate the essential nature of the amino groups of the toxin.

Experimental

The methods for the preparation of the toxin and determination of its toxicity have been described or referred to in the preceding paper.[2]

Inactivation of the Toxin with Nitrous Acid.—Toxin, stored at 5° in *p*H 4.0 acetate, μ 0.05, was mixed with suitable acetate buffers also at *p*H 4.0 to give an acetate concentration of 0.50 *M* and a toxin concentration between 1 and 2.5 mg. per ml. The solution was chilled to 0° and the reaction started by adding an equal volume of 0.67 *M* $NaNO_2$ at the same temperature. The temperature was maintained at 0° ±0.2 in a constant temperature refrigerated bath. Samples of 0.1 ml. were withdrawn at intervals and the reaction stopped by diluting 100-fold in gelatin–phosphate diluent. Each sample was assayed for toxicity immediately after withdrawal to determine the dilution range and again the next day to get a more precise result.

Kinetics of Deamination of Alanine.—Samples of 2.0 ml. of DL-alanine in 0.5 *M* buffer were chilled to 0° and mixed with an equal volume of chilled sodium nitrite solution. The reaction was stopped by adding 4.0 ml. of alkali of sufficient strength to bring the mixture to slightly above *p*H 7. No deamination occurred for at least 2 hr. after the *p*H adjustment. Acetate buffer was used for runs at *p*H 4 and above and citrate for the runs at *p*H 3.5 and 3.75. The *p*H of the buffer–nitrite mixtures was determined at 25° on a Beckman model G *p*H meter.

Determination of Unreacted Alanine.—A 5.0-ml. sample of the neutralized reaction mixture was delivered into the chamber of a Van Slyke manometric apparatus. Gas was removed from the sample by lowering the mercury to the 50-ml. mark, shaking for two minutes and then expelling the gas without allowing any of the sample to enter the cup. A 2.0-ml. portion of a glacial acetic acid–saturated sodium acetate mixture (1:1) was then run into the chamber under mercury. This buffer was previously deaerated in the Van Slyke apparatus and stored over mercury in a container similar to the one designed by Sendroy.[12] Finally, 2.0 ml. of sodium nitrite solution (40 g. dissolved in 50 ml. of water) was added and the chamber sealed with mercury. The mercury level was lowered to the 50-ml. mark and the reaction allowed to proceed for 5 minutes, shaking during the last minute. This prolonged reaction time was necessary because of the buffered solution which diminished the rate of reaction. The gas was then transferred to a Hempel pipet containing alkaline permanganate and the nitrogen measured in the usual manner.[13]

Results with this method were not as accurate as with the original method, but reproducibility was within 1 to 2% with 300 mm. of pressure.

(12) J. Sendroy, Jr., *Ind. Eng. Chem., Anal. Ed.*, **9**, 190 (1937).
(13) D. D. Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGY, THE UNIVERSITY OF ROCHESTER]

Non-enzymic Macromolecules as Matrices in Biological Synthesis: The Role of Polysaccharides in Peroxidase-catalyzed Lignin Polymer Formation from Eugenol

BY S. M. SIEGEL

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Enzymatic peroxidation of eugenol yields several classes of products separable according to solubility and dependent in their formation on reaction conditions. The system peroxidase–H_2O_2–eugenol (aqueous) yields a variety of chloroform and some ethanol-soluble products including dimeric and side-chain degradation products. Introduction into this system of certain macromolecules effected a change in the reaction, adding dioxane- and ethanol–HCl–soluble substances having the elementary composition, ultraviolet spectrum and chromogenic properties of lignins. These macromolecules, regarded as polymerization matrices of low specificity, include celluloses, methylcellulose and chitin. Proteins tested did not act as matrices except for human hair, which yielded anomalous results. Differences in yield and ultraviolet spectra of fractions occur; most notable is the absence of a lignin spectrum in dioxane fractions of water-insoluble matrices. Minimum size for matrices lies between raffinose and arabic acid. Deacetylation increases the efficacy of chitin as a matrix and partial acetylation reduces efficacy of filter paper. The most extreme departure from the polysaccharide matrix, the use of macromolecules such as asbestos minerals and others, still yielded lignin-like products in small amounts.

Although a detailed mechanism for the condensation of phenylpropane units in lignin polymer formation has not been described, the fact of the formation of lignins from members of this wide group of plant substances has been well established.[1,2] The author has been especially concerned with a peroxidative system which forms lignin polymers from eugenol in a few minutes to a few hours.[3] This system, demonstrated in tissue slices and cell walls, was not duplicated by the simple mixture of eugenol, peroxide and crystalline peroxidase, suggesting that wall components could orient eugenol, or an intermediate formed by its peroxidation, so that lignin could be formed.

As a simple means of testing this conjecture, peroxidase-infiltrated filter paper was introduced into eugenol–peroxide systems, and lignin found shortly thereafter to be deposited in the paper.[4] Methylcellulose solutions served as cellulose substitutes but deposited greater amounts of lignin per unit weight of polysaccharide. Further, the distribution of products as based upon solvent fractionation differed in the two cases. These observations have given rise to the concept of a matrix substance which might serve as an orienting surface in the polymerization process. As a corollary to the postulated matrix function, it was recognized that distinction should be made between a template whose surface is able to orient small molecules in a highly ordered array, and, at the opposite extreme, the non-specific adsorption common to such substances as charcoal, alumina or silica gel. The matrix concept implying an intermediate condition of specificity should be applicable to polymerization of additional classes of biochemical substances, and thus would recognize the possible role of non-enzymic macromolecules in cellular

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(2) S. Siegel, *Quart. Rev. Biol.*, **31**, 1 (1956).
(3) S. Siegel, *Physiol. Plant.*, **8**, 20 (1955).
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