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The Reaction of Tobacco Mosaic Virus with Formaldehyde. II. Kinetics[2]

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Introduction

The loss of infectivity of tobacco mosaic virus in the presence of formaldehyde was studied by Ross and Stanley.[4] It appears to be a reaction of the first order. When the inactivation is carried out at room temperature in a phosphate buffer at *p*H 7, a simultaneous decrease in the amino nitrogen content of the virus occurs and the isoelectric point of the virus is shifted toward the acid side.[5] In a previous paper[6] the authors reported that the reaction products of formaldehyde inactivation appear to be electrophoretically homogeneous but to have a different mobility than untreated virus. Therefore, it seemed desirable to study simultaneously from the kinetic point of view the effects of formaldehyde treatment at *p*H 7.0 on the electrophoretic mobility, the amino nitrogen content and the infectivity of the virus to determine the possible relationship between the three changes.

Materials and Methods

Tobacco mosaic virus, isolated by the differential centrifugation method of Stanley,[7] was treated with formaldehyde by allowing a solution of 2% virus and 2% formaldehyde in 0.1 *M* phosphate buffer, *p*H 7.0, to react at 30.0°. At suitable time intervals, 15-ml. aliquots of the reacting mixture were removed and the *p*H immediately lowered to the isoelectric *p*H of the virus (*p*H 3.5) by the addition of 20 ml. of cold 1 *M* phosphate–citrate–hydrochloric acid buffer. After centrifuging for fifteen minutes at 4,000 r.p.m., the clear supernatant fluid was discarded and the virus precipitate washed by suspending the precipitate in 30 ml. of cold 0.1 *M* phosphate–citrate–hydrochloric acid buffer, *p*H 3.5, and again centrifuging and decanting the supernatant fluid. Washing was repeated and the precipitate then was dissolved in 10 ml. of phosphate buffer, *p*H 7.00 ± 0.02, 0.20 ionic strength, and dialyzed for four days against four 250-ml. portions of the same buffer. The virus protein content was determined by micro-Kjeldahl analysis, and the preparations of formaldehyde-treated virus were stored in the refrigerator. The infectivity, amino nitrogen content and the electrophoretic mobility of each sample were then determined.

Infectivity was determined by the method of Loring,[8] using *N. Glutinosa* as the test plant. So that approximately equal numbers of local lesions could be compared, two dilutions of each preparation were made and compared by using the half-leaf method and a Latin-square pattern for inoculation. The ninhydrin method described by Miller and Stanley[9] was used to determine the amino nitrogen content of each sample and electrophoretic mobilities were determined at 1° in the apparatus described by Tiselius[10] and modified by Longsworth.[11] In preparation, the virus had already been dialyzed against phosphate buffer, *p*H 7.00 ± 0.02, 0.20 ionic strength. Therefore, a portion of each sample was diluted with this buffer to a virus concentration of 0.50% just prior to electrophoresis in the same buffer. Differences in the *p*H and the specific conductances of the protein solutions and the buffer were not detectible by the precision bridge described by Luder.[12] Migration in an electric field of 4.5 volts per cm. was allowed to proceed until the essentially homogeneous boundaries had migrated a distance of 6 cm. The current was then reversed and the boundaries brought back to their original positions. The reported mobilities were calculated from the average of the distances each boundary moved away from and back to its original position.

The observation that formaldehyde-treated virus is electrophoretically homogeneous[6] justified the use of isoelectric precipitation to stop the reaction quickly. Table I shows that the method was efficient in removing excess and reversibly bound formaldehyde, for the properties of the virus treated 0-hours with formaldehyde were essentially the same as stock and control virus. Further evidence that excess formaldehyde was probably completely removed was obtained by studying the effect of the presence

TABLE I

USE OF ISOELECTRIC PRECIPITATION TO STOP THE FORMALDEHYDE-TOBACCO MOSAIC VIRUS REACTION

Treatment		Activity remaining, % stock virus	Ninhydrin color, % stock virus	Electrophoretic mobility, sq. cm./volt-sec. × 10^5
(1) Stock virus	(a)	100	100	..
	(b)	100	100	7.17
(2) Isoelectric precipitation	(a)	107	89	..
	(b)	104	100	7.16
(3) 0-hr. 2% HCHO followed by isoelectric precipitation	(a)	116	91	..
	(b)	78.6	101	7.14

TABLE II

THE EFFECT OF SMALL CONCENTRATIONS OF FORMALDEHYDE ON THE NINHYDRIN REACTION

Sample (5 mg. TMV in 1 ml.)	Water added, ml.	HCHO added, ml. of 0.36% HCHO	Concn. HCHO, %	Color[a]
1	0.10	0.00	0.000	Deep blue
2	.08	.02	.007	Deep blue
3	.06	.04	.014	Blue
4	.04	.06	.022	Light blue
5	.02	.08	.029	Faint blue
6	.00	.10	.036	Colorless

[a] Color decreased visibly according to formaldehyde concentration.

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(3) Some sections were abstracted from a thesis submitted by Marie A. Fischer to the Department of Chemistry in partial fulfillment of the requirements for the Ph.D. degree.

(4) A. F. Ross and W. M. Stanley, *J. Gen. Physiol.*, **22**, 165 (1938).

(5) W. M. Stanley, *Science*, **83**, 626 (1936).

(6) M. A. Fischer and M. A. Lauffer, *Arch. Biochem.*, **23**, 291 (1948).

(7) W. M. Stanley, THIS JOURNAL, **64**, 1804 (1942).

(8) H. S. Loring, *J. Biol. Chem.*, **121**, 637 (1937).

(9) G. L. Miller and W. M. Stanley, *ibid.*, **141**, 905 (1941).

(10) A. Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937).

(11) L. G. Longsworth, THIS JOURNAL, **61**, 529 (1939).

(12) W. F. Luder, *ibid.*, **62**, 89 (1940).

of formaldehyde on the ninhydrin reaction. To 1-ml. aliquots of stock virus, containing 5 mg. of tobacco mosaic virus, varying concentrations of formaldehyde were added, and ninhydrin determinations were carried out immediately. It is apparent from the data in Table II that small concentrations of formaldehyde inhibit the ninhydrin reaction and that the immediate reaction of excess formaldehyde affects all of the groups which are reactive to ninhydrin because color is completely absent. However, Table I shows that the immediate reaction can be reversed by the removal of the formaldehyde. This is in contrast to the Van Slyke amino nitrogen reaction which has been shown[13] to reverse partly or completely the immediate binding of formaldehyde by amino groups.

Presentation of Experimental Results

Rate of Inactivation.—Ross and Stanley[4] studied the rate at which infectivity is lost when a 2% solution of virus in 0.1 *M* phosphate buffer at *p*H 7 is allowed to react with 2% formaldehyde at room temperature. The results of similar experiments confirm the observation of Ross and Stanley that the loss of infectivity of tobacco mosaic virus in the presence of formaldehyde is a reaction of the first order. The data fit an equation of the form

$$(V) = e^{-kt} \qquad (1)$$

where (V) is virus infectivity expressed as fraction of original infectivity and t is time of contact with formaldehyde in hours. In four experiments carried out at *p*H 7 and 30°, the following values were obtained for k: 0.37, 0.44, 0.45 and 0.4 reciprocal hour.

Shift in Electrophoretic Mobility with Formaldehyde Treatment.—As was pointed out previously,[6] tobacco mosaic virus remains essentially homogeneous with respect to electrophoretic mobility after treatment with formaldehyde; however, a mobility shift occurs, because inactive virus can be separated electrophoretically from untreated virus. Since treated virus is inhomogeneous with respect to the criterion of biological activity, but homogeneous with respect to electrophoresis, it seemed desirable to study the mobility shift in more detail. In preliminary studies the kinetics of this reaction were studied by an indirect method.[14] It was first shown that a correlation could be established between the infectivity remaining, following treatment, and the electrophoretic mobility. When the logarithm of the relative activity remaining was plotted against the electrophoretic mobility of a formaldehyde-treated sample, a straight line relationship was obtained. If this proportionality is considered in relation to the logarithmic dependence of infectivity upon time, it can be deduced that the shift in electrophoretic mobility is directly proportional to the time of contact between the virus and the formaldehyde.

The experiment described in the section on materials and methods was carried out in a manner which permitted direct measurement of both time of contact with formaldehyde and electrophoretic mobility. The results are shown in Fig. 1, where electrophoretic mobility increase in a 0.1 *M* phosphate buffer at *p*H 7 is plotted as a function of time of contact with formaldehyde. The linear relationship is seen to apply only to the initial stage of the reaction. The open circles on the graph represent experimental determinations, and the smooth curve is a plot of equation (2).

$$\Delta U = 0.82(1 - e^{-0.04t}) \qquad (2)$$

ΔU in equation (2) represents the increase in anodic mobility, and t represents the time in hours during which the virus was held in contact with formaldehyde.

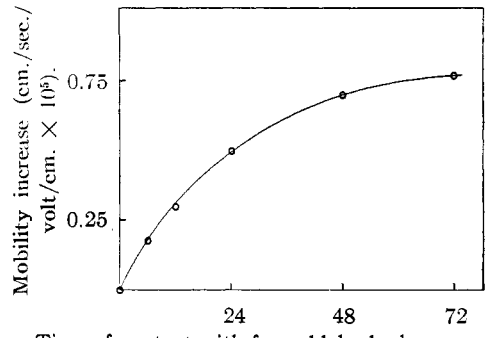

Fig. 1.—Increase in anodic electrophoretic mobility (10^5 cm.^{2}/volt-sec.) of formaldehyde-treated tobacco-mosaic virus protein in potassium phosphate buffer, *p*H 7.00 ± 0.02, 0.2 ionic strength, plotted against time in hours of reaction at *p*H 7.0 and 30° between the protein and 2% formaldehyde.

The Decrease in Free Amino Groups.—Ross and Stanley,[4] by measuring the ninhydrin color, studied the effect of formaldehyde treatment in neutral phosphate buffer on the virus groups. In order to determine whether or not the decrease in free amino groups as shown by the ninhydrin reaction is correlated with the shift in mobility or with the decrease in infectivity, free amino groups were measured on the samples which were subjected to infectivity and electrophoretic analyses. The details of the experiment were described in the section on materials and methods. The results are presented in Fig. 2, where ninhydrin color expressed as per cent. of original is plotted against time in hours of reaction with formaldehyde. The open circles represent the experimental values obtained. If the shift in electrophoretic mobility were associated in a simple manner with the decrease in amino nitrogen, these data ought to fit the equation, $C = 100e^{-0.04t}$, where C is the color produced by ninhydrin expressed as per cent. of original, and t is the time in hours of reaction with formaldehyde. The data do not obey this equation at all. This is proof that ninhydrin does not measure exactly the

(13) H. S. Olcott and H. Fraenkel-Conrat, *Chem. Revs.*, **41**, 151 (1947).

(14) M. A. Lauffer and M. A. Fischer, preliminary report of this investigation presented to the Division of Biological Chemistry of the American Chemical Society at New York City, 1947.

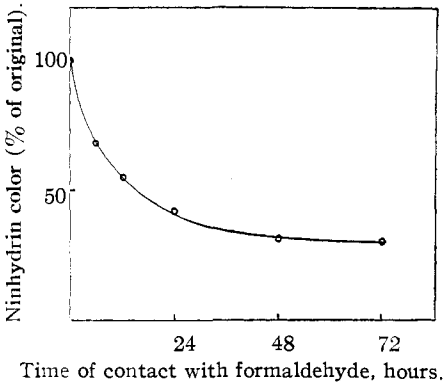

Fig. 2.—Color, expressed as per cent. of original absorption, obtained by treating formaldehyde-modified tobacco-mosaic virus protein with ninhydrin plotted against time in hours of reaction at *p*H 7.0 and 30° between the protein and 2% formaldehyde.

same thing as shift in electrophoretic mobility. The smooth curve in Fig. 2 is a plot of equation (3).

$$C = (28 + 30e^{-0.04t} + 42e^{-0.14t}) \quad (3)$$

The parameter, 0.04, was carried over from equation (2) for reasons which will be discussed later. The other four parameters were selected to make the equation fit the data. The parameter, 28, is subject to an error of the order of magnitude of 3%. The other parameters are subject to uncertainties of the order of magnitude of 25%.

Discussion

Theoretical Considerations.—Assume that a solution contains n macromolecules per ml., each with ν groups capable of reacting with a reagent. The total concentration of groups per ml. will be $n\nu$ or N. If the reagent is present in great excess, one would expect the reaction to be first order with respect to the groups.

$$-dN/dt = kN \quad (4)$$

$$N/N_0 = e^{-kt} \quad (5)$$

N/N_0 is the fraction of groups unchanged after a time, t, of reaction with the reagent.

$$1 - N/N_0 = N'/N_0 = 1 - e^{-kt} \quad (6)$$

N'/N_0 is the fraction of the groups which changed. The theory of probability leads to the expectation that the product should be inhomogeneous with respect to the number of groups per macromolecule reacted after time, t. The mean should be $\nu N'/N_0$. The distribution should be described by a standard deviation, σ.

$$\sigma = \sqrt{\nu N N'/N_0^2} \quad (7)$$

Suppose that m of these ν groups on each macromolecule have some special significance. Then

$$n/n_0 = e^{-mkt} \quad (8)$$

where n/n_0 is the fraction of macromolecules on which no one of the m special groups has reacted after time, t, in contact with reagent. The rate of disappearance of macromolecules with m intact special groups would be given by equation (9).

$$-dn/dt = mkt \quad (9)$$

Types of Reaction between Formaldehyde and Proteins.—The possible reactions between formaldehyde and protein were reviewed in detail by French and Edsall[15] and by Olcott and Fraenkel-Conrat[13]. Apparently, formaldehyde is able to add reversibly to free amino groups to give groups which are weak bases and which do not produce color with ninhydrin. Further, apparently irreversible, reactions are possible by cross-linking between amino methylol groups and amide, guanidyl, imidazole, indole and phenol groups. These irreversible reactions may or may not lead to a change in charge, but do lead to change in color production with ninhydrin.

Shift in Mobility.—The negative charges on tobacco mosaic virus protein arise largely from free carboxyl groups of the dicarboxylic amino acids. These would be almost completely ionized at *p*H 7 because the pK is of the order of 4.0.[16] The negative charges are probably partially neutralized internally by positive charges originating from guanidine residues of arginine and the epsilon amino groups of lysine. At *p*H 7 the guanidine groups should be completely ionized; the free amino groups of lysine should be ionized to the extent of about 99.7%, since the pK for such groups in the polypeptide state is about 9.5, or greater.[16] Calculation from the amino acid content of tobacco mosaic virus[17] leads to the conclusion that at neutrality a tobacco mosaic virus particle should have 8,300 net charges.[18]

The mobility shift observed as a result of the reaction of the virus with formaldehyde can be interpreted as being the result of a decrease in the number of positive groups per particle, thereby increasing the net negative charge. The increase in negative charge, therefore, should be given by the quantity $\nu(N'/N_0)(\alpha_1 - \alpha_2)$, where α_1 and α_2 are the dissociation constants of the groups before and after reaction with formaldehyde, respectively. When the expression for N'/N_0 given by Equation (6) is substituted into this quantity, when it is considered that the maximum change in

(15) D. French and J. T. Edsall, *Adv. Protein Chem.*, **2**, 277 (1945).

(16) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids, and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943.

(17) C. A. Knight, *J. Biol. Chem.*, **171**, 297 (1947).

(18) Unpublished acid–base binding experiments indicated that 6900 moles of acid were bound by one mole of untreated tobacco mosaic virus protein and 8200 moles of acid were bound by one mole of formaldehyde inactivated virus protein when the proteins were titrated from *p*H 3.5 (the usually accepted isoelectric point of tobacco mosaic virus protein) to *p*H 7.0. These data indicate that the theoretical valence, −8300, is of the correct order of magnitude. It should be observed that nucleic acid was omitted from consideration in arriving at the theoretical valence. This amounts to the assumption that nucleic acid does not contribute appreciably to the net charge of tobacco mosaic virus protein at *p*H 7. The reasonable agreement between the theoretical valence and that obtained by titration experiments indicates that this assumption may not be seriously in error.

charge is equal to $\nu(\alpha_1 - \alpha_2)$, and when the assumption is made that the shift in electrophoretic mobility is directly proportional to the shift in net charge, Equation (10) results.

$$\Delta U = \Delta U_{\max.} (1 - e^{-kt}) \qquad (10)$$

It is obvious that this equation is of exactly the same form as empirical Equation (2).

Experiments reported in a previous publication[6] showed that, following reaction with formaldehyde for a twenty-four-hour period, tobacco mosaic virus exhibited boundaries in the electrophoresis apparatus which were about as sharp as those exhibited by untreated virus. However, the theoretical considerations summarized by Equation (7) lead one to expect that virus treated with formaldehyde should be inhomogeneous with respect to electrophoresis. On the assumption that mobility is equal to K times the charge, Equation (11), where σ_u is the standard deviation of the distribution of mobility increments, can be derived from Equation (7).

$$\sigma_u = K \sqrt{\nu \frac{N'}{N_0} \times \frac{N}{N_0}} \qquad (11)$$

Since there are approximately 8,300 excess negative charges on a tobacco mosaic virus particle at *p*H 7, and the mobility is 7.15×10^{-5} sq. cm./volt-sec., K can be evaluated to be 0.00086×10^{-5}. Chemical analyses indicate that there are about 3,400 free amino groups on a tobacco mosaic virus particle. For reasons which will be discussed in the next section, about 30% of these can be assumed to be involved in the reaction which leads to a change in ionization. One can infer from Fig. 1 that N'/N_0 and N/N_0 are both approximately one-half for the reaction which has continued for twenty-four hours. By substituting these values into Equation (11), one can estimate that the standard deviation of the increment in mobility due to reaction of tobacco mosaic virus with formaldehyde should be of the order of magnitude of 0.014×10^{-5} cm./sec./volt/cm. Since the mobility after twenty-four hours of reaction is about 7.5×10^{-5}, the ratio of the standard deviation of the increment in mobility to the mobility is about 0.002. Inspection of electrophoresis boundary diagrams obtained with untreated tobacco mosaic virus indicates that the ratio of the standard deviation of the boundary to distance traversed is of the order of magnitude of 0.01. Therefore, the standard deviation of the boundary obtained with treated virus ought to be $\sqrt{(0.01)^2 + (0.002)^2}$, a value about 2% greater than that obtained with untreated virus. A difference of this magnitude would go undetected. Thus, the experimental observation that virus after reaction with formaldehyde is still essentially homogeneous with respect to electrophoresis is not inconsistent with the concepts used in the present case to interpret the reaction mechanism.

Decrease in Free Amino Groups.—The data presented in Fig. 2 were obtained under such conditions that they represent the irreversible change in free amino groups resulting from reaction of tobacco mosaic virus with formaldehyde. If one makes the assumption that there are three kinds of amino groups on tobacco mosaic virus particles, (a) those which do not react irreversibly with formaldehyde; (b) those which react irreversibly with formaldehyde at a relatively rapid rate; and (c) those which react with formaldehyde at a relatively slow rate, then the fraction of free amino groups remaining after time, t, would be given by a function which is the sum of a constant and two exponentials like that of Equation (5). The curved line fitting the data in Fig. 2 is a graph of Equation (3), and Equation (3) is of exactly the form described.

If the assumption is made that one of the two kinds of groups which react with formaldehyde irreversibly leads to the shift in electrophoretic mobility illustrated by the data of Fig. 1, and that the other of the two kinds of groups does not result in a shift in mobility, then it is necessary that the constant in one of the exponential terms of Equation (3) be the same as the constant in the exponential in Equation (2). Thus, the parameter, 0.04, in Equation (3) was determined from the data of Fig. 1 and not from those of Fig. 2.

It is thus evident that the results of these studies are consistent with the assumption that at least three kinds of amino groups are present on a tobacco mosaic virus particle. About 28% of the groups do not react irreversibly with formaldehyde; about 42% react irreversibly with a rate constant of about 0.14 but do not lead to a change in charge; and about 30% react irreversibly with a rate constant of 0.04 to produce a change in charge. The data of Fig. 2 are not sufficiently precise to fix these parameters with any great precision nor to rule out the possibility that more than three kinds of groups are present.

The data of Fig. 1 can be interpreted to mean that the maximum shift in mobility at *p*H 7 due to reaction of tobacco mosaic virus with formaldehyde is 0.82×10^{-5} sq. cm./volt sec. If this is divided by K, a value of 953 is obtained for the increase in net negative charge, or the decrease in positive charge per virus particle. If it is assumed that the change in charge involves free amino groups, and that $\alpha_1 = 1$ and $\alpha_2 = 0$, the shift of 953 charges would involve 953/3400 or 28% of the free amino groups. This figure agrees with the parameter, 30, in Equation (2) and constitutes evidence in favor of the reasonableness of the assumption that 30% of the amino groups react irreversibly with formaldehyde to produce a change in charge.

Kinetics of the Decrease in Infectivity.—The data of the present study and of previous studies indicate that the decrease in infectivity obtained when tobacco mosaic virus is treated with formaldehyde is a reaction of the first order. This means that the inactivation process, what-

ever it is, must be a single event phenomenon. Two possibilities exist for interpreting the decrease in infectivity. One is that the reaction is completely independent of any changes detected by chemical or electrophoretic means. The other possibility is that infectivity is lost when the first one of several particular amino groups on a virus particle has reacted irreversibly with formaldehyde. If several groups are capable of leading to inactivation, they must all be of the same sort with respect to rate of reaction with formaldehyde; otherwise, the first order law for inactivation could not be followed. If one assumes that m out of a total of ν groups of a particular sort on a virus particle can lead to loss in infectivity when irreversible reaction with formaldehyde takes place, then loss of infectivity would result when the first of these groups happened to react. Equation (9) shows that the reaction velocity constant for the loss of infectivity would be equal to m times the velocity constant for the reaction of the particular groups under study.

The reaction velocity constant for the destruction of infectivity at room temperature has been shown to be about 0.42 reciprocal hour. This is about ten times the rate constant for the change in mobility. Thus, if groups of the sort which result in shifts in mobility are responsible for the inactivation of the virus, there must be ten special groups of that sort on each virus particle. Similarly, since the rate of the reaction which leads to irreversible loss of free amino groups but not to change in charge is 0.14, there would have to be 0.42/0.14 or 3 special groups of this sort to account for the loss of infectivity.

Summary

The kinetics of the changes which take place when tobacco mosaic virus is treated with 2% formaldehyde at *p*H 7 and 30° were studied. Infectivity was found to decrease according to the law of a first order process with a rate constant of about 0.42 hour^{-1}. Electrophoretic mobility was found to increase and approach a maximum value as the time of treatment was extended indefinitely. The rate constant for this process was found to be 0.04 hour^{-1}. Free amino groups as determined by the ninhydrin color reaction were found to decrease according to a complex pattern. The results can be interpreted in terms of the assumption that 28% of the amino groups do not react irreversibly with formaldehyde; 42% react irreversibly at a rate of 0.14 reciprocal hour, and 30% react irreversibly at a rate of 0.04 reciprocal hour. These latter 30% can be assumed to be the same groups which cause shift in electrophoretic mobility. The loss of infectivity could be due either to some process entirely independent of the reactions indicated by the chemical and physical changes or to the first one of several particular amino groups of one sort reacting irreversibly with formaldehyde.

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[CONTRIBUTION FROM THE RICHARDSON CHEMICAL LABORATORY, TULANE UNIVERSITY]

A New Synthesis of Tuberculostearic Acid[1a]

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Tuberculostearic acid or 10-methyloctadecanoic acid (compound V in Fig. 1) was isolated by Anderson and Chargaff[2] from the fatty envelope surrounding the tubercle bacillus and its structure later proved by Spielman[3] who prepared a synthetic sample. Recently Prout, Cason and Ingersoll[4] have reported preparation of the *dl*-form and the *d*- and *l*-enantiomorphs of 10-methyloctadecanoic acid, establishing that the naturally occurring isomer is the levorotatory form. The *dl*- and active forms have also been prepared by Ställberg-Stenhagen[5] by a still different method.

As a part of a study of derivatives of modified branched-chain fatty acids as potential antitubercular chemotherapeutic agents, we have undertaken the preparation of moderate amounts of tuberculostearic acid to be used in further synthetic work. We have developed a new method of synthesis of *dl*-tuberculostearic acid which appears to be an improvement over the one used by Spielman.[3] It is much shorter than the synthesis used by Prout, Cason and Ingersoll[4] and Ställberg-Stenhagen,[5] since these authors prepared the *d*- and *l*-forms which involved working with optically active intermediates and avoiding racemization in the transformations employed.

The steps in this synthesis are outlined in Fig. 1. Azelaic acid was converted to its half ethyl ester acid chloride and this was allowed to react with 2-decylzinc chloride (II), to give ethyl 9-keto-10-methyloctadecanoate (III). Reduction of the keto ester (III) by the Clemmensen method gave ethyl 10-methyloctadecanoate (IV) which was hydrolyzed to the corresponding acid (V). Purification of 10-methyloctadecanoic acid was effected by converting it to the amide (VI) followed by recrystallization of the amide and hydrolysis to the acid.

A distinctive feature of this synthesis is the use

(1a) Presented before the Organic Division, Atlantic City A. C. S. meeting, Sept. 21, 1949.

(1b) Frederick G. Cottrell, Research Fellow, 1948–1949.

(2) Anderson and Chargaff, *J. Biol. Chem.*, **85**, 77 (1929).

(3) Spielman, *ibid.*, **106**, 87 (1934).

(4) Prout, Cason and Ingersoll, THIS JOURNAL, **70**, 298 (1948).

(5) Ställberg-Stenhagen, *Arkiv Kemi, Mineral. Geol.*, **26A**, No. 12 (1948), 28 pp.