From our results with PBLG preparations it would appear that formic acid possesses no unique properties with respect to $\alpha \rightarrow \beta$ transformations, but rather that it is a good solvent for low molecular weight (β)-polypeptides and a poor solvent for high molecular weight (α)-polypeptides. It may be possible, of course, that the differences between our results with formic acid treatment of PBLG, and those of the previous workers who used D,L-polypeptides for the most part, are due to the greater stability of the α-helix when only amino acid residues of one optical configuration are involved. This matter is under investigation and will be reported shortly.

The $\beta \rightarrow \alpha$ Transformation.—It has been reported[6] that certain polypeptides may be made to undergo a $\beta \rightarrow \alpha$ transformation by heating to 280° *in vacuo*. The conversion from $\beta \rightarrow \alpha$ was determined by both X-ray and infrared determinations. On heating cast films of our PBLG polypeptides in a similar manner to that described, we observe that typical spectral changes indicated in Fig. 9—namely, that the original material, which was a mixture of α and β, *on heating to 280°* and cooling to room temperature, appears to be predominantly α. However, from the intensities of the infrared bands and the weights of the samples before and after heating, it is apparent that there is *a loss of β-material* from the sample (by sublimation) *and no increase in the amount of the α-material.* Therefore no actual $\beta \rightarrow \alpha$ change has occurred and it is suggested that the previously reported $\beta \rightarrow \alpha$ transformation may have the same explanation.

In view of these studies it may be concluded that the *configuration of PBLG in the solid state is dependent on the molecular weight* and not on the prior solvent treatment or thermal history of the polypeptide. The infrared spectral results reported above with preparations of poly-γ-benzyl-L-glutamate are representative of those obtained with several other polypeptide polymers and copolymers which results will be published in due course. These data, from synthetic polypeptides of known composition and molecular weight, will be related to infrared data from proteins.

Acknowledgment.—We gratefully acknowledge the support by the Office of the Surgeon General, Department of the Army, and the assistance of Mr. R. H. Karlson and Miss E. Smakula in the preparative aspects of this work.

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[CONTRIBUTION FROM THE ARTHRITIS RESEARCH LABORATORY OF THE BOSTON DISPENSARY AND THE CANCER RESEARCH AND CANCER CONTROL UNIT OF THE DEPARTMENT OF SURGERY, TUFTS UNIVERSITY SCHOOL OF MEDICINE]

"Enzymoid" Properties of Lysozyme Methyl Ester

BY EDWARD H. FRIEDEN[1]

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Conditions are defined under which an "enzymoid," an inactive enzyme derivative which retains affinity for substrate, can be expected to inhibit the activity of the native enzyme. Lysozyme methyl ester has been found to inhibit the lytic activity of lysozyme, and the data are consistent with the assumption that competition between enzyme and enzymoid for substrate occurs. Approximate K_m values for lysozyme–substrate and methyllysozyme–substrate interaction are 1.1 and 0.015, respectively. The implication of these findings for the problem of the relationship of structure to biological activity of proteins is discussed.

A large body of information is available concerning the effects of chemical alteration upon enzymes and other biologically active proteins. Usually, the effects of such alterations have been followed by measurement of changes in the activity of the protein being studied, sometimes correlated with quantitative estimates of the extent of chemical change. These studies have established the relationship between activity and specific structural features for a variety of enzymes and protein hormones.[2] However, the question of just what part a group which has been found to be essential plays in enzyme or hormone function has generally gone unanswered.

In this paper there will be outlined a method by means of which the function of essential groups of some enzymes can be defined, and its application to lysozyme will be described.

According to current concepts, an enzymatically catalyzed reaction proceeds through the initial formation of an enzyme–substrate complex, which then decomposes into reaction product(s) and free enzyme. If it is assumed that the affinity of enzyme for substrate and its catalytic property are functions of different parts, or at least different structural features, of the molecule, it follows that inactivation of an enzyme can occur in either of two possible ways: (1) modification or destruction of groups which are necessary for combination of enzyme and substrate, or (2) alteration of a structural element, the integrity of which is necessary for catalytic activity, *i.e.*, the decomposition of the enzyme–substrate complex into enzyme and reaction products. It is further readily apparent that specific modifications corresponding to type (2) would give rise to a derivative which would retain the characteristic affinity of the native enzyme for its substrate, but which would be devoid of catalytic activity. Under appropriate conditions, such a derivative might be expected to inhibit the apparent activity of the native enzyme by competing with the latter for available substrate. It should

(1) Guggenheim Foundation Fellow, University of California at Los Angeles, 1953–1954. Presented in part at the April, 1955, Meeting of the Federation of American Societies for Experimental Biology, San Francisco, California.

(2) R. R. Porter, in "The Proteins," H. Neurath and K. Bailey, eds., Academic Press, Inc., New York, N. Y., 1953, Vol. I, part B, pp. 973–1015.

be noted that such a situation would be the obverse of competitive inhibition as the term is conventionally employed. Instead of the inhibition being due to competition between substrate and inhibitor for attachment to enzyme, there would be involved competition between native and modified *enzyme* for combination with substrate.

Many years ago, Bayliss[3] offered evidence that heat-inactivated trypsin preparations retained their affinity for substrate; he proposed the generic term "zymoid" for inhibitors of this kind. Bearn and Cramer[4] studied the effects of several heated enzyme preparations on the corresponding native enzymes. Inhibition frequently was observed, but was attributed to the existence of preformed "zymoids" in the original preparations, their presence having been unmasked by destruction of the active enzyme. The subject was reopened many years later by Yagi,[5] who suggested the term "enzymoid" and reported unsuccessful attempts to prepare inhibitors of taka-diastase and crystalline urease, again by heat inactivation.

Although it is theoretically possible to prepare derivatives with enzymoid properties from any enzyme, the experimental demonstration of inhibition of the native enzyme by a suitably modified derivative imposes an important restriction on the choice of a test system. This restriction arises from the fact that inhibition will be manifested only when an appreciable proportion of the total substrate is bound by the inhibitor *under conditions such that the reaction velocity is substrate-dependent.* Brief reflection will suffice to show that there is required an enzyme–substrate system such that the reaction velocity be measurable and substrate-dependent at relatively low substrate concentrations.

For example, consider an enzymatically catalyzed reaction the rate of which is linearly proportional to substrate concentration at concentrations of the latter above 10^{-2} *M*. To inhibit the reaction rate by 25% (*i.e.*, to reduce the free substrate concention by 25%) there would be required an inhibitor (modified enzyme) concentration in excess of 2.5×10^{-3} *M*; this situation corresponds, for an enzyme of molecular weight 50,000, to an "enzymoid" concentration of 125 g./l. In addition to possible solubility problems, the specificity of inhibitions found with protein concentrations of this order of magnitude would be open to serious question.[6]

For a suitable test system, the lysis of *M. lysodeikticus* by lysozyme was selected. The rate of clearing of a suspension of killed cells is proportional to enzyme concentration up to a concentration of 0.020 mg. lysozyme/ml., and, at enzyme concentrations within this range, the velocity is first order with respect to substrate concentration when the latter is varied from 0.2–1.0 mg./ml. A wide choice of derivatives is available, since lysozyme is sensitive to a number of different kinds of chemical agents.[7] One disadvantage is the fact that precise definition of the substrate concentration in molecular terms is not possible, since the substrate is macromolecular. However, if it is assumed that the mucopolysaccharide substrate of lysozyme, which is present in the cell at a concentration of about 20% of its dry weight,[8] possesses an equivalent weight of at least 1,000, it can be calculated that significant inhibition is to be expected at inhibitor concentrations of about 3 g./l. As will be shown later, the use of a macromolecular, sedimentable substrate offers some advantages which tend to offset its apparent disadvantages.

The ready availability of lysozyme in a state of high purity, the abundance of information concerning its constitution and properties, and the simplicity of measurement of its lytic action, were other factors contributing to its selection.

Experimental

Crystalline lysozyme was purchased from the Armour Laboratories. Lysozyme methyl ester was prepared essentially according to the procedure described by Fraenkel-Conrat.[7] Three hundred milligrams of lysozyme was dissolved in 30 ml. of a solution prepared by adding 0.8 ml. of concd. HCl to 100 ml. of absolute methanol. A gelatinous precipitate formed rapidly. The suspension was allowed to stand at room temperature (22–24°) for 24 hours,[9] then diluted with water and dialyzed for 24 hours against several changes of cold distilled water, after which the impermeate was lyophilized. Two hundred milligrams of product was obtained. Electrophoretic analyses in TRIS buffer (*p*H 8.15, ionic strength 0.05) showed that approximately 95% of the protein moved as a single peak, with a mobility appreciably faster than native lysozyme under these conditions.[10] Under conditions such that 50% lysis occurs in 2 min. with a lysozyme concentration of 0.0067 mg./ml., no perceptible clearing occurred during 20 min. with 0.0625 mg./ml. of the methylated derivative.

Titration data showed that of the 8.5 acid groups found per mole (14,500 g.) of native lysozyme, 5.2 were esterified under the conditions described above. These data were in excellent agreement with the results of quantitative methoxyl analyses,[11] which indicated the presence of 5.3 methoxyl groups per mole of methyl lysozyme.

M. lysodeikticus was grown on nutrient agar in Roux bottles. After 48 hours at 37° the cells were harvested, exposed to phenol for 1 hr., washed and lyophilized.

Activity Measurements.—Sodium phosphate buffer, ionic strength 0.15, *p*H 7.0 was used as the solvent. Optical density was measured with a Klett–Summerson colorimeter, filter #540. For most of the experiments reported here, a solution of methyl lysozyme was added to substrate suspended in buffer and the optical density recorded during the ensuing 3–4 min. Native enzyme was then added and the optical density measured at intervals of 30–60 sec. during the first 2–3 min., and at less frequent intervals thereafter. In other experiments, a freshly-prepared mixture of native and modified enzyme was added to substrate at zero time, and readings taken at intervals as indicated above. The two methods gave generally concordant results. To minimize the effects of temperature changes while readings were being taken, the water-bath in which the reaction mixtures were incubated was set at 27.5°, slightly above room temperature.

(3) W. M. Bayliss, "The Nature of Enzyme Action," Longmans, Green & Co., New York, N. Y., 1911, 2nd ed.

(4) A. R. Bearn and W. Cramer, *Biochem. J.*, **2**, 174 (1907).

(5) Y. Yagi, *Symposia on Enzyme Chem. (Japan)*, **2**, 7 (1949); *C. A.*, **46**, 4755 (1951).

(6) Since dependence of reaction velocity upon substrate concentration may generally be expected within about one log unit above and below K_M (the Michaelis constant), an approximate upper limit for K_M would appear to be in the range 10^{-3}–10^{-4}. It is possible that Yagi's failure to observe inhibition with heated urease was in part due to the fact that K_M for urease is 2.5×10^{-2} (E. A. Moelwyn-Hughes in "The Enzymes," J. B. Sumner and K. Myrbäck, eds., Academic Press, Inc., New York, N. Y., 1950, Vol. I, part 1, pp. 58); the corresponding enzymoid concentration for 10% inhibition would be in excess of 10 grams per cent.

(7) H. Fraenkel-Conrat, *Arch. Biochem.*, **27**, 109 (1950).

(8) K. Meyer and E. Hahnel, *J. Biol. Chem.*, **163**, 723 (1946).

(9) Shorter reaction periods led to incomplete inactivation.

(10) I am indebted to Dr. Peter Bernfeld for the electrophoretic analyses.

(11) Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

The discussion of results which follows is based upon experiments in which the lysozyme concentration was 0.0067 mg./ml. Parallel experiments with a lower enzyme concentration (0.0033 mg./ml.) afforded comparable results.

Results

The result of a typical experiment is shown in Fig. 1, in which extent of hydrolysis is plotted against time for constant enzyme and substrate concentration in the presence of a series of concentrations of lysozyme methyl ester. In Fig. 2 is summarized the effects of the methyl derivative in experiments in which the initial substrate concentration was varied from 0.25 to 1.00 mg./ml. For each inhibitor concentration, the reaction velocity (calculated from the initial slope of the corresponding rate curve) is expressed as the percentage of that found in the absence of inhibitor.

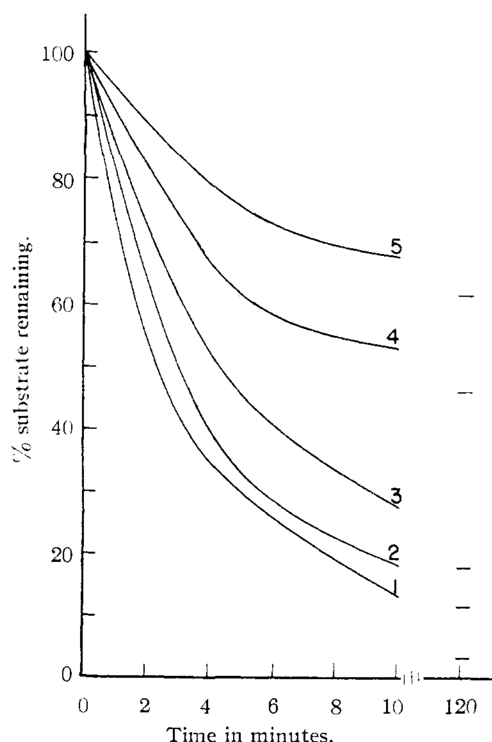


Fig. 1.—The effect of increasing concentrations of methyllysozyme upon lysis of *M. lysodeikticus* by lysozyme. Concn. of lysozyme = 0.0067 mg./ml.; initial concn. of substrate = 0.50 mg./ml. Methyllysozyme concentrations: curve 1, 0; curve 2, 0.025 mg./ml.; curve 3, 0.050; curve 4, 0.075; curve 5, 0.100.

It is clear from Figs. 1 and 2 that methyllysozyme does in fact inhibit the lytic action of the native enzyme. It may be seen, upon inspection of Fig. 2, that the concentration of methyllysozyme necessary for inhibition of the initial reaction velocity by 50%, for example, is proportional to substrate concentration; this fact indicates that noncompetitive inhibition of the enzyme is not involved. The possibility that the observed inhibition is due to competition between methyllysozyme and substrate for the reactive sites of native lysozyme is not, however, ruled out by the observed facts, however unlikely such a mechanism may appear. Indeed, as Friedenwald and Maengwyn-Davies have pointed out,[12] the behavior of an inhibitor which binds substrate in competition with enzyme, and that of one which combines with enzyme in competition with substrate are so similar as to be distinguishable only by kinetic experiments at extremely low inhibitor concentrations, where departure from linearity of conventional Lineweaver–Burk plots may be expected. Fortunately, since the substrate is macromolecular and sedimentable, a simple means of distinguishing between these two alternatives is available.

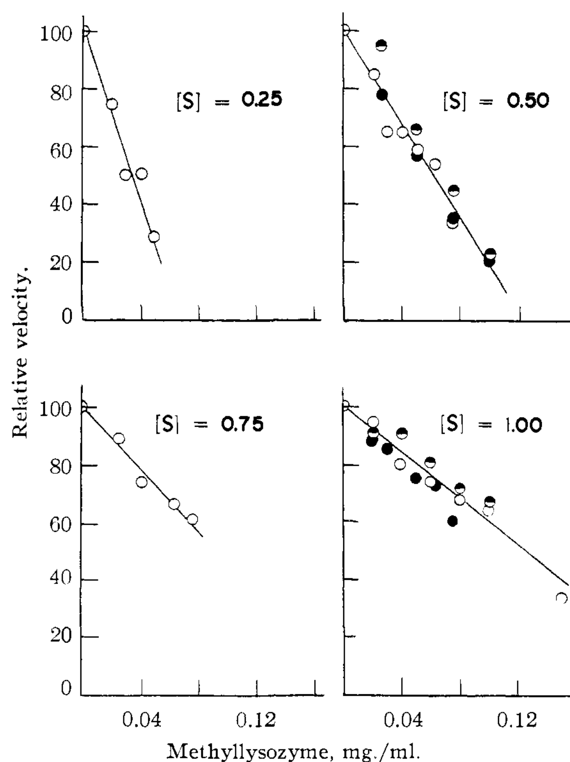


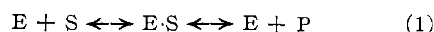
Fig. 2.—Effect of methyllysozyme upon initial reaction rate. Concn. of enzyme = 0.0067 mg./ml. Initial substrate concentration [S] in mg./ml. is given on the graph; each symbol represents a different experiment.

Mixtures of *M. lysodeikticus* cells suspended in buffer and methyllysozyme in various proportions were prepared, incubated 10–15 min. at 27.5°, and the cells removed by centrifugation. The concentration of protein in the clear supernatant solution was determined by measuring its absorption at 2800 Å., the values so found being corrected for material which may have been leached out of the cells. Under these conditions, the concentration of methyllysozyme which remained in solution was invariably found to be below the limits for precise measurement. At the highest inhibitor:substrate ratio tested (0.45:1), less than 20% of the former was unbound.

From this experiment it is clear that methyllysozyme possesses a high affinity for substrate. The data are consistent, therefore, with the assumption that a system consisting of substrate (S), lysozyme

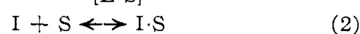
(12) J. S. Friedenwald and G. D. Maengwyn-Davies, in "The Mechanism of Enzyme Action," W. D. McElroy and B. Glass, eds., The Johns Hopkins Press, Baltimore, Md., 1954, p. 165.

(E), and methyllysozyme (I) is adequately described by the following equilibria



$$E + S \leftrightarrow E{\cdot}S \leftrightarrow E + P \quad (1)$$

$$K_1 = \frac{[E][S]}{[E{\cdot}S]} \quad (1a)$$



$$I + S \leftrightarrow I{\cdot}S \quad (2)$$

$$K_2 = \frac{[I][S]}{[I{\cdot}S]} \quad (2a)$$

K_1 is most conveniently evaluated by use of the Lineweaver–Burk form of the Michaelis–Menten equation

$$V_m/V - 1 = K_1/[S] \quad (3)$$

where V is the measured velocity for substrate concentration [S] and V_m the maximum attainable velocity for the particular enzyme concentration used. V_m was estimated by plotting V against 1/[S]. By plotting ($V_m/V - 1$) *vs.* 1/[S], estimates of 1.0 and 1.2 mg./ml. for K_2 were obtained for enzyme concentrations of 0.0067 and 0.0033 mg./ml., respectively.[13]

To estimate K_2, it is assumed that the effect of the inhibitor is to reduce the reaction velocity by lowering the concentration of free substrate. From equation 2 it can be shown that the free substrate concentration is related to total substrate present [S_t] and free inhibitor [I] by

$$[S] = \frac{[S_t]}{1 + [I]/K_2} \quad (4)$$

Substituting for [S] in equation 3 its equivalent from equation 4, and rearranging

$$\frac{V_m}{V} - 1 = \frac{K_1}{[S_t]} + \frac{K_1[I]}{K_2[S_t]} \quad (5)$$

Thus, if $V_m/V - 1$ is plotted against inhibitor concentration (total S being constant), a straight line of slope $K_1/K_2[S_t]$ and intercept $K_1/[S_t]$ will result. K_2 can be evaluated as the ratio of intercept to slope.

The similarity of equation 5 with the corresponding relation between relative reaction rate and concentration of a competitive inhibitor in the conventional sense may be noted. In contrast to the situation which usually obtains in experiments with competitive inhibitors, however, it cannot be assumed that the concentration of methyllysozyme bound to substrate is negligible in comparison with the total present. Some means of estimating the concentration of free I must therefore be found in order that equation 5 may be applied. While it is possible to derive a unique expression for [I] in terms of K_2, [I_t] and [S_t],[12] it is unmanageable in its complexity. An alternative method is available, however. For any point on the lines of Fig. 2, the concentration of free substrate can be calculated from the measured reaction rate as the substrate concentration which gives this same rate in the absence of inhibitor. The concentration of bound substrate is calculated by difference. Finally, if an estimate can be had of the combining ratio of I:S in the substrate–inhibitor complex, bound I, and thus free I can be calculated, [I_t] being known.

The I:S ratio was estimated by first calculating the apparent ratio for several values of [I_t] at each substrate concentration, extrapolating to zero I, and then plotting the extrapolated values so found against 1/[S_t] and extrapolating again to 1/[S_t] = 0. The average value found for the two enzyme concentrations was 0.16.

A representative point-by-point calculation of free I for one set of data ([S_t] = 0.50 mg./ml., [E_t] = 0.0067 mg./ml.) is shown in Table I; the corresponding plot of $V_m/V - 1$ *vs.* [I] is illustrated in Fig. 3, together with the calculation of K_2.

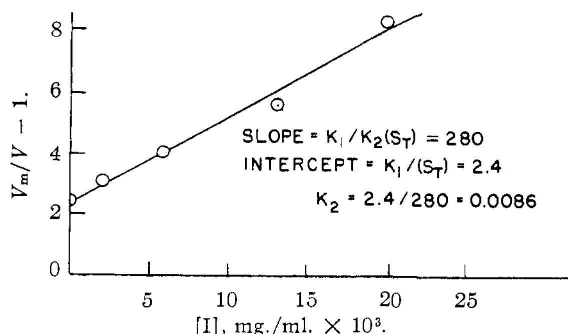


Fig. 3.—Sample plot of ($V_m/V - 1$) *vs.* (I), and calculation of K_2 from data of Table I.

TABLE I

CALCULATION OF UNBOUND INHIBITOR CONCENTRATION

Inhibitor added (mg./ml.)	Obsd. reaction rate (mg./ml./min.)	[S] (mg./ml.)	Bound [S] (mg./ml.)	Bound [I] (mg./ml.) (bound [S] × 0.16)	[I] (mg./ml.)
0.00	0.116	0.50	0.00	0.00	0.00
.020	.097	.39	.11	.018	.002
.040	.078	.29	.21	.034	.006
.060	.060	.21	.29	.047	.013
.080	.041	.13	.37	.060	.020

The values of K_2 calculated by the method outlined above for the various sets of data available range from 0.007 to 0.028. The calculated values are in satisfactory agreement, considering the approximations involved. It may be concluded that lysozyme methyl ester is bound some 50–100 fold more tightly to the reactive sites of the substrate than is the native enzyme.

It will be noted (Fig. 1) that in the presence of moderate concentrations of methyllysozyme, a substantial proportion of substrate remains unchanged after prolonged incubation. For example, upon the addition of 0.075 mg./ml. of modified enzyme, approximately 45% of the substrate is left after 2 hours, although the initial reaction rate is about one-third that in the absence of inhibitor. If the mixture is observed for periods as long as 12–14 hrs., the amount of further lysis which occurs is insignificant. This phenomenon has been observed in some degree for all reaction mixtures containing the inhibitor; the amount of unreacted substrate depends upon the amount of inhibitor present, and is roughly independent of the initial substrate concentration. The unreacted residue would appear to represent the inhibitor-bound substrate remaining

(13) The use of a macromolecular substrate of unknown equivalent weight makes it necessary to introduce the assumption that however many reactive sites exist on each bacterial cell, all are identical. This assumption appears justified in view of the close adherence of the reaction to first order kinetics. The equilibrium constants are expressed in weight rather than molecular concentrations.

after all the free substrate has been lysed. The apparent irreversibility of the formation of IS in the presence of relatively small concentrations of enzyme is consistent with the relative magnitudes of K_1 and K_2 as calculated above. For the concentrations which pertain to the example cited ($[I_t]$: $[E_t] = 11$) it would be expected that the rate of lysis would decrease by a factor of 500–1000 when nearly all the free substrate has disappeared.

Discussion

The experiments reported here indicate that lysozyme methyl ester, although devoid of lytic activity, retains the affinity of the native enzyme for its substrate. However, before concluding that the carboxyl groups of lysozyme are important for its catalytic action, but are not required for formation of the intermediate enzyme–substrate complex, it must be shown that there has occurred no change in the molecule other than the methylation of some of the carboxyl groups. That inactivation was not due to denaturation by acid or methanol was established by control experiments in which solutions of lysozyme in neutral methanol and in dilute aqueous acid were allowed to stand 24 hours, then assayed. No loss in activity was observed for either solution.

Probably the simplest and most convincing demonstration of the specificity of the action of acid methanol would be to show that the reaction is reversible, *i.e.*, that lytic activity is restored upon removal of the methoxyl groups. Attempts to saponify methyllysozyme by prolonged exposure to cold dilute acid or alkali (conditions which do not affect the native enzyme) were unsuccessful in restoring enzymatic activity; whether part or all of the methoxyl groups were actually removed by this treatment is not known. Further work on this phase of the problem, and experiments with other carboxyl-specific alkylating agents, will be needed before the specificity of the effect observed can properly be assessed.

The increase in affinity for substrate which occurs when lysozyme is converted to its methyl ester ($K_1 = 1.1$; $K_2 = 0.01$–0.02) deserves some comment. If the attachment of lysozyme to the reactive site of the cell surface involves electrostatic forces between anionic groups of the substrate and cationic groups of the enzyme (a possibility suggested by the high isoelectric point of lysozyme) the presence in the enzyme of neighboring carboxylate residues would have the effect of diminishing the net positive charge in the region of the enzyme concerned, and thus of reducing the attractive force between enzyme and substrate. Elimination of the charge on the carboxyl group by conversion to its methyl ester would have the opposite effect, resulting in increased affinity of enzyme for substrate, as was actually observed.

For its successful application, the method described above depends upon a suitable Michaelis constant as a characteristic of the enzyme concerned, and upon the availability of specific (and preferably reversible) reagents for the functional groups of protein molecules. Within these limitations, it seems capable of providing much useful information regarding the relationship between structure and function. The qualitative demonstration of enzymoid (*i.e.*, inhibitory) properties associated with a derivative prepared by altering or removing a particular functional group would indicate that this group is not required for combination of enzyme and substrate, and that its essential nature is presumably related to its role in the catalytic process. Quantitative comparison of native and modified enzyme, as well as of derivatives in which the same group has been attacked by different reagents could be expected to yield information from which further details of structure-function relationship might be inferred.

It should be noted that the possibility of differentiating between various aspects of the function of biologically active molecules by selective inactivation is by no means confined to enzymes. The well-known toxin–toxoid relationship (whence, probably, the term enzymoid) represents the application of the same principle to a different class of biologically active proteins. Similar considerations suggest that appropriately modified protein hormones might be capable of inhibiting the native hormone.

Acknowledgment.—The author wishes to record his indebtedness to Dr. T. A. Geissman for many stimulating discussions of this problem.

BOSTON, MASS.