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18 February 1966

Enzyme Action: Comparison on Soluble and Insoluble Substrate

Abstract. *The action of trypsin on gelatin solution is compared with its action on swollen gel microspheres in suspension. Both the solution gelatin and gel spheres, which are readily permeable to the enzyme, follow Michaelis-Menten kinetics. The apparent rate constants for dissociation of the enzyme-substrate complexes to hydrolysis products are essentially the same for both solution gelatin and spheres, an indication that gel structure in this system has a negligible influence on reaction rate once the enzyme forms a complex with the substrate. In contrast, the Michaelis constant for the gel system is greater than that for solutions below the melting point of the gel; this difference disappears as the melting point of the gel is approached.*

Despite the observed ubiquity of enzyme action on insoluble substrates in nature (*1*), most kinetic studies have concerned only the action of enzymes on soluble substrates. A rigorous comparison of enzyme action on both soluble and insoluble forms of the same substrate is complicated by the fact that one cannot, in general, find two distinct equilibrium states (soluble and insoluble) or phases of a single substrate under the same physical conditions, except at phase transitions, where both forms coexist. Thus, the elaboration of the kinetics of enzyme action on each of two separate forms or phases of the same substrate requires that at least one of these forms be a nonequilibrium configuration under the prevailing conditions.

The most satisfactory situation is one in which both soluble and insoluble forms arise naturally as a consequence of hysteresis of the substrate with respect to variation of one or more thermodynamic variables. In this event substrate molecules of identical chemical composition and molecular weight comprise both forms. In the comparison, reported by Mazia and Hayashi (*2*), of the action of pepsin in pepsin-ovalbumin fibers and on free heat-denatured ovalbumin it is probable that the substrate molecules do not have the same chemical composition and molecular weight in both soluble and insoluble forms. Attempts to disperse fibers of ovalbumin in familiar protein solvents, including the potent combination of high *p*H, 8*M* urea, and excess mercaptoethanol (which completely disperses irreversibly aggregated heat-denatured ovalbumin), were uniformly unsuccessful. Thus, it must be presumed that covalent intermolecular bonds are present in the fibers.

Ordinary gelatin is a suitable substrate because, upon cooling sufficiently concentrated solutions, it readily forms gels which are essentially stable against dispersion into excess solvent, and which may be redissolved by mild heating. However, in studying the kinetics of enzymatic digestion of a gel it is desirable to employ volumes of gel sufficiently small so that the enzyme

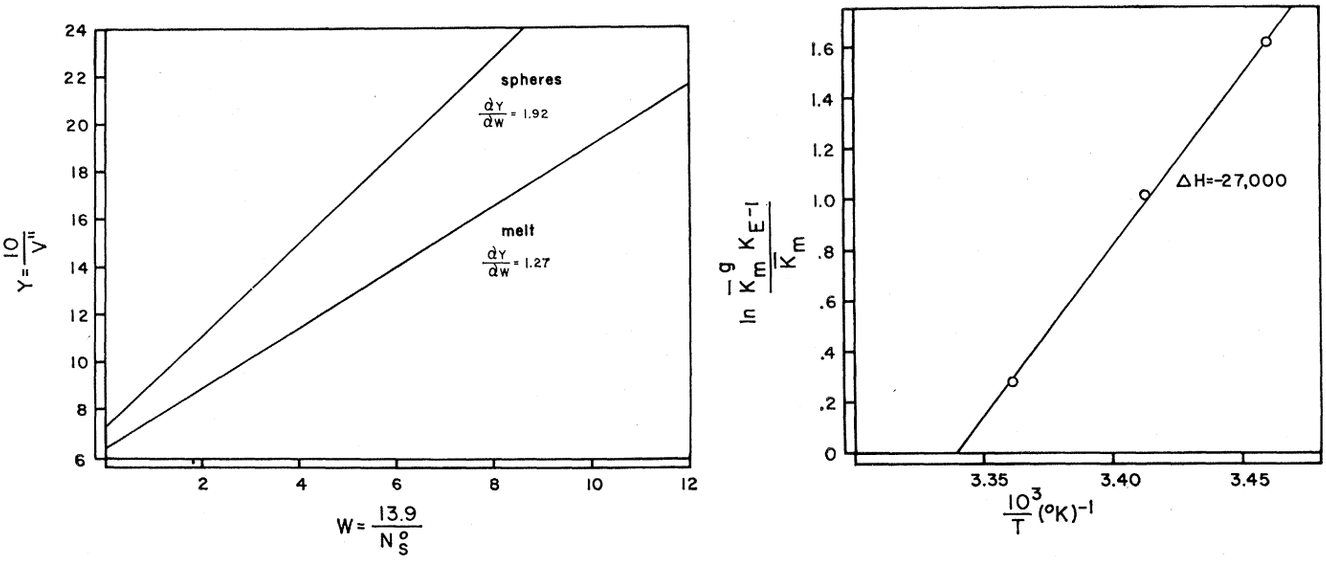

Fig. 1 (left). For both spheres and free-solution gelatin, $10/V''$ is plotted against W which is equal to $13.9/N_S^0$. Here V'' is the observed initial rate in units of syringe volume (percent) delivered per minute, and the value for W is a reciprocal factor for the dilution of a stock suspension (13.9 mg/ml) to N_S^0. The trypsin concentration is $7.5 \times 10^{-11}M$ in both situations. The syringe contained 0.01*N* NaOH, and the solution was 0.15*M* KCl in both cases; *T* 24.5°C; *p*H, 8.90. Fig. 2 (right). The unusual temperature dependence of $\bar{K}_m^g K_E^{-1}$ is completely exposed after factoring out the temperature dependence of the ordinary free solution constant K_m. The ordinate represents ln $\bar{K}_m^g K_E^{-1}/\bar{K}_m$ and the abscissa represents $10^3/T$, where T is the absolute temperature.

could, if unhindered, acquire access to all regions within the gel in a time short compared to both (i) the duration of the kinetic experiment and (ii) the time required to digest the gel sufficiently to permit dispersion into the surrounding solvent. Gel microspheres between 10 and 100 μ in diameter may be prepared by rapidly cooling a coacervate mixture of warm, dilute gelatin and ammonium sulfate (*3*). The excess gelatin and ammonium sulfate may be removed by centrifugation and washing. The estimated time for the enzyme trypsin to diffuse across spheres of this size has been shown to be very much less than the duration of the kinetic experiment or digestion time of the gel (*4*).

The action of trypsin on gelatin microspheres in suspension is here compared with its action on dilute gelatin sols which were obtained by melting out a corresponding suspension of gel spheres. The kinetics of the reaction were followed with a *p*H stat. Absolute rates were obtained from titration curves of the α-amino groups liberated in the reaction and by titrating the trypsin with soybean trypsin inhibitor to determine the concentration of active trypsin molecules. By following the digestion of gelatin microspheres with a phase contrast microscope we observed that the digestion of substrate proceeded uniformly throughout the interior with no decrease in sphere diameter (*3*). This observation is in contrast to that of Tsuk and Oster (*5*), who reported a steady decrease in the dimensions of a disulfide cross-linked gel (thiogel) when immersed in a trypsin solution. Furthermore, the hydrolysis rates of both our microspheres and gelatin sols were proportional to the trypsin concentration also in contrast to the result of Tsuk and Oster, who found a rate proportional to the 0.77 power of the trypsin concentration. Both the apparent impermeability of the disulfide cross-linked gel and the 0.77 power dependence of the rate on enzyme concentration strongly implicate surface adsorption of the Freundlich type in the action of trypsin on that gel (*6*).

The more familiar behavior of our permeable gel spheres was further borne out by the dependence of the rate on substrate concentration. The total gelatin concentration in the sphere suspensions was varied simply by changing the concentration of spheres. As can be seen in Fig. 1, the rate for both microspheres and gelatin sols gives a linear Lineweaver-Burk plot. Thus the rate (V) data may in both instances be described by an equation of the form:

$$V = K_A N_E^0 N_S^0/(K_B + N_S^0) \qquad (1)$$

where N_E^0 is the total trypsin concentration, N_S^0 is the initial gelatin concentration, and K_A and K_B are empirically determined constants. The absolute rate V (moles of peptide hydrolyzed per minute) is related to the observed initial rate of base consumption V'' (Fig. 1) by an appropriate constant factor (*4*). The relation between K_A, K_B, and fundamental physical constants of the system may be established by analysis of a suitable model. If there are n susceptible bonds per milligram of gelatin and these are divided into $r<n$ classes, each class being characterized by a K_3 and K_m, and if each class is in a steady-state, then it may readily be shown (*4*) for free solution gelatin that

$$K_A = \bar{K}_3 \equiv \left\langle \frac{K_3}{K_m} \right\rangle \left\langle \frac{1}{K_m} \right\rangle^{-1}$$

and

$$K_B = \bar{K}_m \equiv \frac{1}{n} \left\langle \frac{1}{K_m} \right\rangle^{-1},$$

where the brackets denote averages over all bonds in all classes. If a particular class of bond predominates, as might be expected for gelatin in which a preponderance of tryptic attack between lysine and glycine has been found (*7*), then $\bar{K}_3$ and $\bar{K}_m$ reduce to the appropriate constants characterizing that class.

With the gel spheres it is necessary to allow the possibility of an equilibrium partition of the free enzyme between the bulk phase and the gel phase. It can be shown (*4*) for our gel spheres that the constants K_A^g and K_B^g determined from Eq. 1, where g designates gel, may be closely approximated by $K_A^g = \bar{K}_3^g$ and $K_B^g = \bar{K}_m^g K_E^{-1}$, where $K_E = N_E^g/N_E^S$ is the equilibrium constant for partition of unbound enzyme between outside solution (concentration N_E^S) and the interior of the gel (concentration N_E^g). Both $\bar{K}_3^g$ and $\bar{K}_m^g$ represent the same kinds of average quantities as $\bar{K}_3$ and $\bar{K}_m$, although the fundamental constants employed are those characterizing a gelatin molecule in the gel.

Reasons, based on theory, have been given for the use of reactant concentrations in the rate expressions for solutions that are as far from thermodynamic ideality as are the microsphere suspensions (*3*).

It was found that $\bar{K}_3$ was essentially the same as $\bar{K}_3^g$, both constants increasing from about 320 moles of peptide hydrolyzed per minute per mole of active trypsin at 16°C to 1000 moles at 24.5°C. This result implies that the substrate structure in the gel has a negligible influence on the reaction rate once the enzyme forms a complex with the substrate. This is a significant observation and not merely a manifestation of a general insensitivity of $\bar{K}_3$ to substrate structure, because very large changes in $\bar{K}_3$ have been found accompanying structural transitions in free-solution gelatin (*4*).

$K_m^g K_E^{-1}$ is larger than $\bar{K}_m$ by a factor of 1.3 at 24.5°C, and this factor increases with decreasing temperature. We may write $K_m^g K_E^{-1}$ as

$$\left(\frac{\bar{K}_m^g K_E^{-1}}{\bar{K}_m}\right) \bar{K}_m$$

Now, although $\bar{K}_m$ decreases sharply with decreasing temperature, as expected for an "effective" dissociation constant, $\bar{K}_m^g K_E^{-1}$ actually increases from 20° to 16°C, an indication that the normal temperature dependence of of the dissociation constant $\bar{K}_m$ has been overcome by a contrary temperature dependence of the factor $\bar{K}_m^g \bar{K}_E^{-1}/\bar{K}_m$ which varies exponentially with $1/T$ (Fig. 2). The factor's dependence on temperature must lie in either the ratio $\bar{K}_m^g/\bar{K}_m$ or K_E^{-1} or both. It may be shown that the principal factor in $\bar{K}_m^g K_E^{-1}/\bar{K}_m > 1$ cannot be a $K_E^{-1} > 1$. If K_E^{-1} is greater than 1, this would indicate a repulsion or exclusion of the enzyme from the gel spheres; repulsion could only arise from either electrostatic repulsion or physical occlusion of the enzyme. The former possibility may be eliminated, since at the *p*H (8.90) of the experiments trypsin is positively charged (isoelectric point, 10.8), whereas the gel microspheres are negatively charged or neutral (*4*). Furthermore, the negative ΔH observed is characteristic only of electrostatic attraction (*4*). The second possibility may be eliminated owing to the observed permeability of the spheres to the enzyme and the failure to observe accelerations in the reaction rate as the spheres are opened up by the enzyme. Thus, we may conclude that the principal factor in $\bar{K}_m^g K_E^{-1}/\bar{K}_m$ is not a $K_E^{-1} > 1$, but is $\bar{K}_m^g/\bar{K}_m > 1$. Our interpretation

is that $K_P^{-1} = 1$, and that the gelatin in the spheres either bears a tertiary structure which must be disrupted to facilitate binding of the enzyme or is associated to form small "crystalline" regions which are impermeable to the enzyme, and which increase in extent as the temperature is lowered. Thus the amount $\bar{K}_m^g$ of substrate required to form a complex with half of the enzyme (in the gel phase) will be greater than $\bar{K}_m$ and will increase with decreasing temperature. A fact which is nicely consistent with this interpretation is that $\bar{K}_m^g K_E^{-1}/\bar{K}_m$ approaches in a continuous manner the value 1.0 near 27°C, close to the melting point of the gel (28° ± 1°C). A discontinuity in $\bar{K}_m^g K_E^{-1}/\bar{K}_m$ would result if either $\bar{K}_m^g/\bar{K}_m$ or K_P^{-1} differed appreciably from 1.0 at the melting point. We conclude that the differences in structure between free-solution and gel-state gelatin tend to vanish smoothly as the melting point of the gel is approached.

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10 January 1966

Multiple Sclerosis: Serum Factor Producing Reversible Alterations in Bioelectric Responses

Abstract. *Serums obtained from patients during acute exacerbations of multiple sclerosis produce a reversible depression of polysynaptic reflex responses when applied to the isolated spinal cord of the frog. Motoneuron discharges initiated by monosynaptic activation through an axosomatic spinal pathway are much less affected than reflex discharges. The active factor in serum appears to depend on the presence of complement.*

Auto-immunity processes have long been suspected to play an important part in the etiology of multiple sclerosis. As early as 1934, Sachs and Steiner claimed, on the basis of an extensive study, that complement-fixing antibrain antibodies could be detected in the serum of patients with this disease (*1*). Although their conclusions were challenged from time to time by other investigators, a strong body of immunological evidence for the presence of circulating auto-antibodies has accumulated since (*2*). In keeping with the hypothesis of an auto-immune pathogenesis for multiple sclerosis, a number of neuropathological analogies have been described between the demyelinative lesions of the central nervous system observed in patients and the lesions produced in animals with experimental "allergic" encephalomyelitis (*3*). More recently, this view received additional support from the demonstration, in the serums from patients (*4*) and animals affected with the experimental disease (*5*), of complement-dependent factors inducing similar patterns of demyelination in cultured fragments of mammalian central nervous tissue.

However, the precise relationship between the immune processes, demyelinative lesions, and clinical symptoms of multiple sclerosis is still far from understood. Indeed, it is usually recognized that the sudden and transient attacks followed by prolonged remissions, particularly during the initial stages of the disease, cannot be accurately correlated with lesions in the central nervous system and be entirely accounted for by the process of demyelination alone (*6*). Therefore, the finding of a circulating factor directly responsible for functional disorders in central nerve cell-bodies or fibers would be of particular significance in elucidating the pathogenetic mechanisms of the disease. In fact, in vitro experiments relevant to this problem have recently been reported by Bornstein and Crain (*7*). These authors found that serums obtained from two patients, during acute exacerbations, produced within a few minutes marked alterations in complex electrical responses of cultured cerebral and spinal cord tissues of the mouse. Of particular interest was the observation that these reversible alterations, resulting presumably from interference with synaptic processes, were due to a complement-dependent serum factor and occurred long before any morphological changes were detected in the cultured fragments.

In the experiments described here, the isolated spinal cord of the frog (*Rana temporaria*) was used to investigate the effects of serum from patients with multiple sclerosis on bioelectric responses of a highly organized nerve center. The preparation was mounted in a recording chamber and submitted to a constant flow of circulating Ringer solution maintained at 10°C. The Ringer solution, equilibrated with oxygen, had the following composition (in millimoles per liter): NaCl, 112; KCl, 2; $CaCl_2$, 1.8; $NaHCO_3$, 2.4; glucose, 26. The cord was allowed to stabilize in this solution for 2 hours after dissection. Dorsal and ventral roots were suspended in air on stimulating and recording platinum electrodes. Motoneuron discharges were recorded from the ninth or tenth ventral root; they were evoked either monosynaptically, by stimulation of lateral column fibers establishing axosomatic connections, or through polysynaptic reflex pathways, by stimulation of the ipsilateral dorsal root (*8*). Mean values of peak amplitude of discharge were calculated from at least ten oscilloscope recordings initiated at 3-second intervals. Serums obtained from normal humans and from patients were used, at the concentration of 40 percent, to prepare solutions whose Na, K, Ca, and glucose contents were adjusted to duplicate the composition of the Ringer solution. The preparation was removed from the recording chamber and transferred to beakers for exposure to these media, kept at 10°C, and equilibrated with oxygen. To facilitate the access of applied solutions, the spinal cord was hemisected sagittally in the experiments dealing with the monosynaptic responses, whereas it was sectioned transversely above and below the lumbar enlargement when the segmental reflex activity was investigated.

A series of control experiments showed that exposure to serum from normal humans sustained or even en-