THE DIFFUSION CONSTANT OF PECTINMETHYLESTERASE

by

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TABLE OF CONTENTS

INTRODUCTION

Foreword

This attempt at a determination of the diffusion constant was prompted by a desire to learn something of the size and shape of the enzyme molecule, pectinmethylesterase.

An electrophoretic study and a determination of the isoelectric point have been made by McCulloch (9). Other than this, however, physicochemical work has not been done with this substance.

The method of determining the diffusion constant used in this research is the scale method developed by Lamm (6) who used the electrophoresis assembly developed by Tiselius.

In addition to the diffusion constant, a value for the molecular weight is estimated by assuming the sedimentation constant to be 1.9×10^{-13} Svedberg units.

Work of this kind is invaluable in furthering the knowledge of size and structure of protein molecules. It helps give a more complete picture of these least known giant molecules.

History of Pectinmethylesterase

This enzyme (1) was discovered in 1840 by Fremy who called it pectase. He prepared it by the alcoholic precipitation of plant juices. Later Davison and Willman obtained active preparations from clover juice and extracts of pollen, and Mehlitz obtained a pectase preparation of very high activity by pressing

the juice from lucerne. A readily soluble enzyme preparation was prepared by Paul and Gransseigne who precipitated the enzyme with acetone in the presence of an added, soluble, colloidal material. These workers also described a superior method of extracting the enzyme using two to four times as much water as the weight of the ground material and allowing the mixture to macerate 36 to 48 hours with subsequent filtration.

Further work has been done by Kertesz (5) who is responsible for changing the name of the enzyme from pectase to pectinmethoxylase and later to pectinmethylesterase. The pH of optimum activity has been determined by Kertesz, and this has been compared by him with the percentage of demethylation occurring under definite conditions. By subtracting the values given by a pectase free solution under the same conditions, an activity curve showing a maximum at pH 6.5 was obtained (1).

Action of Pectinmethylesterase on Pectin

Pectinmethylesterase derived its name from the fact that it catalyzes the removal of the methyl ester group of pectin.

Pectin has been the subject of intensive investigation by many workers. The results have been summarized in two books (1), (2). It seems fairly certain that pectin is a polyuronide composed of α-D-polygalacturonic acid residues of pyranose form combined in 1-4-glycoside linkage (3). This structure is shown in Fig. 1. The minimum size of the polygalacturonide chain is six to eight units. Many investigators have confirmed the

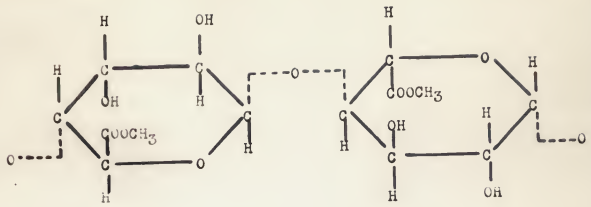


Fig. 1. The structure of pectin.

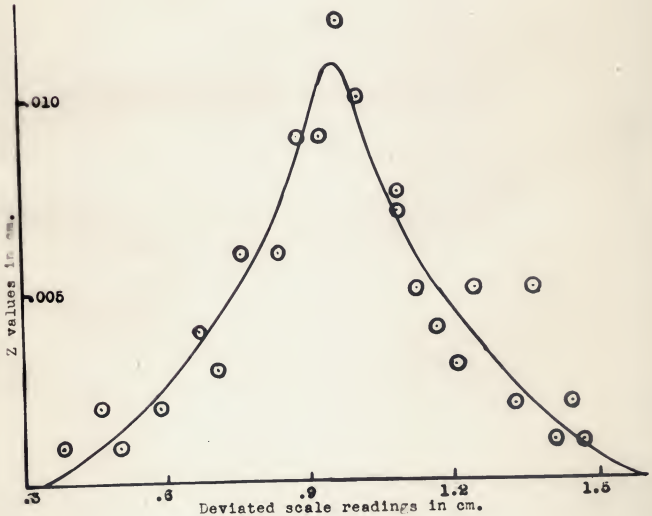


Fig. 2. A diffusion curve.

presence of galactose, arabinose, and polygalacturonic acid in pectin, so the existence of these substances in the molecule seems certain. Just how they fit in with the above structure, however, is still open to question.

Apparently the action of pectinmethylesterase is to catalyze the removal of the methyl ester groups so that pectin is converted into pectinic and pectic acids.

DIFFUSION CONSTANT THEORY

In 1855 Fick stated the general law of free linear diffusion, known today as Fick's first law (7), (11). The law states that the quantity of a substance which will diffuse across an area is proportional to the difference in concentration, the time of diffusion, the cross sectional area, and the concentration gradient. If dm is the amount of solute, $\frac{dc}{dx}$ the concentration gradient, dt the time of diffusion, and q the cross sectional area, then

$$dm = - Dq\frac{dc}{dx}dt. \qquad (1)$$

D is called the diffusion constant. The minus sign simply means that diffusion takes place in the direction of decreasing concentration. Fick formulated this law as a purely empirical relationship, but it has since been shown to be derivable from osmotic theory. D represents the amount of solute that will diffuse across unit area with unit concentration gradient in unit time, provided the rate remains constant.

Since the equation contains two independent variables, x

and t, and two dependent ones, m and c, it is very difficult to use in this form. If the number of variables is reduced by one, the second law of Fick is obtained as follows: Consider an infinitesimal volume of space bounded by planes x and x + dx. Then the amount of solute which will accumulate in this volume in time dt will be the difference between the amount entering across plane x and the amount leaving across plane x + dx. Mathematically stated

$$dm - (dm + \frac{\partial(dm)}{\partial x}dx) = -\frac{\partial(dm)}{\partial x}dx.$$

qdx would represent the volume across which the solute is diffusing, so $\frac{-\frac{\partial(dm)}{\partial x}dx}{qdx}$

represents the concentration increase of solute in that volume. The concentration increase is also $\frac{\partial c}{\partial t}dt$ so that

$$\frac{\partial c}{\partial t}dt = -\frac{\frac{\partial(dm)}{\partial x}dx}{qdx}$$

$$= -\frac{1}{q}\frac{\partial(dm)}{\partial x} \quad \text{or} \quad \frac{\partial(dm)}{\partial x} = -\frac{\partial c}{\partial t}dtq$$

Differentiating Fick's first law with respect to x and substituting the above equation into it gives

$$\frac{\partial(dm)}{\partial x} = -Dq\frac{\partial^2 c}{\partial x^2}dt,$$

$$-\frac{\partial c}{\partial t}dtq = -Dq\frac{\partial^2 c}{\partial x^2}dt$$

or
$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2}. \qquad (2)$$

This expression is Fick's second law. For the special case of

solutions without finite boundary, the following requirements must be met. Actually all physical solutions meet these requirements.

1. $c = c(x, t)$.

2. When $t = 0$, $c = f(x)$.

3. When $t > 0$, $f(x)$ can be differentiated.

A general solution for Fick's second law of diffusion may be arrived at by assuming

$$C = X(x)\,T(t). \tag{3}$$

Use of this makes possible a separation of variables in equation (2). If (3) is differentiated once with respect to t, and twice with respect to x, the result is:

$$\frac{\partial c}{\partial t} = X(x)\,T'(t)$$

and $$\frac{\partial^2 c}{\partial x^2} = X''(x)\,T(t).$$

These may be substituted into Fick's second law,

$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2},$$

to give the result

$$X(x)\,T'(t) = DT(t)\,X''(x),$$

from which the variables may be separated to give

$$\frac{T'(t)}{DT(t)} = \frac{X''(x)}{X(x)}.$$

This equation is true only when each side is equal to the same constant, $-k^2$. Setting each side equal to $-k^2$ gives

$$T'(t) = -k^2 DT(t)$$

and $$-\lambda(x)k^2 = X''(x).$$

These two differential equations have the solutions

$$T(t) = \alpha e^{-k^2Dt},$$

and $X(x) = A\cos kx + B\sin kx$ respectively, in which , A, B, and k are real constants. Since any sum of solutions is also a solution, if the two above solutions are added and integrated over all positive values of k, and, in addition, A and B are taken as functions of k, a new solution results.

$$c = \int_0^\infty \left[g(k)\cos kx + h(k)\sin kx\right] e^{-k^2Dt}dk. \qquad (4)$$

It is necessary to see whether g(k) and h(k) can be so chosen that the second boundary condition (when t = 0, c = f(x)) is fulfilled. That is

$$f(x) = \int_0^\infty \left[g(k)\cos kx + h(k)\cos kx\right] dk.$$

This is found to be the case, according to Fourier, when

$$g(k) = \frac{1}{\pi}\int_{-\infty}^{+\infty} f(\alpha)\cos\alpha k d\alpha,$$

$$h(k) = \frac{1}{\pi}\int_{-\infty}^{+\infty} f(\alpha)\sin\alpha k d\alpha.$$

When these values are substituted into (4) the following equation results:

$$c = \frac{1}{\pi}\int_0^\infty e^{-k^2Dt}dk\left[\int_{-\infty}^{+\infty} f(\alpha)\cos\alpha k d\alpha\cos kx + \int_{-\infty}^{+\infty} f(\alpha)\sin\alpha k d\alpha\sin kx\right]$$

This can be simplified using the formula

$$\cos(\alpha \pm \beta) = \cos\alpha\cos\beta \mp \alpha\sin\beta\sin$$

from Pierce's Integral Tables. The result is

$$c = \frac{1}{\pi}\int_0^{\infty} e^{-k^2Dt}dk\left[\int_{-\infty}^{+\infty} f(\alpha)\cos k(\alpha - x)d\alpha\right].$$

This may be rearranged giving

$$c = \frac{1}{\pi}\int_0^{\infty} f(\alpha)d\alpha\int_{-\infty}^{+\infty} e^{-k^2Dt}\cos k(\alpha - x)dk,$$

which can be integrated using the formula

$$\int_0^{\infty} e^{-a^2x^2}\cos bx\,dx = \frac{\sqrt{\pi}}{2a}\, e^{-b^2/4a^2}$$

letting $a^2 = Dt$, $k = x$, and $\alpha - x = b$. The following equation is the result.

$$c = \frac{1}{2\sqrt{\pi Dt}}\int_{-\infty}^{+\infty} f(\alpha)\; e^{-(\alpha - x)^2/4Dt}d\alpha.$$

It is now necessary to introduce a new variable y, such that $y = \frac{\alpha - x}{2\sqrt{Dt}}$.

Then $\alpha = 2y\sqrt{Dt} + x$ and $d\alpha = 2\sqrt{Dt}dy$.

Substituting these values for α and $d\alpha$ gives

$$c = \frac{1}{\sqrt{\pi}}\int_{-\infty}^{+\infty} f(x + 2y\sqrt{Dt})e^{-y^2}dy. \qquad (5)$$

This equation holds only for diffusion which is not restricted by the dimensions of the cell in which it is taking place. In order for this to be true, the time must be short enough and the cell large enough so that concentration changes do not occur at the extreme ends of the cell. This result (5) is the general solution of the diffusion equation.

When a solute diffuses from a solution into pure solvent, the solute concentration in the solution half of the cell can

be represented as c_o and in the solvent half it will be zero at the beginning of the experiment. These restrictions are the boundary conditions for this case and can be represented mathematically as follows:

$$f(\alpha) = 0, \text{ for } \alpha < 0 \text{ at } t = 0,$$

$$f(\alpha) = c_o, \text{ for } \alpha > 0 \text{ at } t = 0,$$

where α is a real constant representing the distance from the initial boundary.

Substituting these boundary conditions into equation (4) gives as a result.

$$c = \frac{1}{\sqrt{\pi}} \int_0^{\frac{-x}{2\sqrt{Dt}}} 0 \cdot e^{-y^2} dy + \frac{1}{\sqrt{\pi}} \int_{\frac{-x}{2\sqrt{Dt}}}^{\infty} c_o e^{-y^2} dy = \frac{1}{\sqrt{\pi}} \int_{\frac{-x}{2\sqrt{Dt}}}^{\infty} c_o e^{-y^2} dy$$

and

$$c = \frac{1}{\sqrt{\pi}} \int_0^{\infty} c_o e^{-y^2} dy + \frac{1}{\sqrt{\pi}} \int_0^{\frac{x}{2\sqrt{Dt}}} c_o e^{-y^2} dy = \frac{c_o}{2} \int_0^{\frac{x}{2\sqrt{Dt}}} e^{-y^2} dy$$

when 0 and c_o are substituted for $f(\alpha)$. Making the reverse substitutions for y and dy:

$$c = \frac{c_o}{2} + \frac{c_o}{2\sqrt{\pi Dt}} \int_0^{\frac{x}{2\sqrt{Dt}}} e^{-(\alpha - x)^2/4Dt} d\alpha. \qquad (6)$$

This solution represents the area under the family of curves, $e^{-(\alpha - x)^2/4Dt}$, affected by a constant factor, when α assumes values between 0 and $\frac{x}{2\sqrt{Dt}}$. These curves all have the shape of a probability or normal error curve, in which the maximum ordinate decreases to unity as the time approaches infinity. The value of D can be calculated from this equation if the time elapsed and a pair of concentration values are known.

Williams and Cady (11) and Andrews (7) list seven conditions

which must be fulfilled in order for a diffusion experiment to be accurate. They are:

1. Change in concentration during an experiment should be small.

2. There should be no decomposition, association, or chemical reaction of the components of the system.

3. The diffusion constant must be independent of the concentration of the diffusing material, if Fick's law is to be used.

4. There must be a sharp initial interface.

5. Any disturbance or temperature gradient which would cause streaming or mixing must be avoided.

6. There must be no volume change during the experiment.

7. There must be available methods for accurate concentration determinations.

The first six conditions are met by most protein solutions in a good thermostat bath. The optical method developed by Lamm (6), which uses refractive index to measure concentration, is used to fulfill the seventh condition. The method has the advantages of convenience and a high degree of accuracy. Also it is not necessary to disturb the diffusing system in order to follow concentration changes. Optical methods, also, are applicable to many solutions to which methods such as electrical or analytical methods are not applicable, or at least are difficult (7).

Lamm's method of measuring concentration from refractive

index is derived from a method Foucault (12) used for testing telescope mirrors. In this method he screened off a telescope and viewed the image of a net in the mirror to be tested. The arrangement was such that light from the net was reflected from a corresponding part of the mirror, so that localized errors in the mirror caused irregularities in the net reflection.

The next stage in the development of the method was made by Wiener who adapted this use of refraction of light for the measurement of concentration gradients. This adaptation by Wiener, as explained by Lamm (6) in his thesis, is reproduced here.

The refractive index (7) is assumed to be exactly proportional to the concentration, that is $c = Dn$. If this equation is differentiated and substituted into Fick's diffusion equation (2) the result is

$$\frac{\partial n}{\partial t} = D \frac{\partial^2 n}{\partial x^2} , \qquad (7)$$

where n is the refractive index, t is the time, and x the distance of diffusion.

In a diffusion experiment the boundary conditions which must be satisfied are:

1. $x = 0$, at $t = 0$,
2. $n = n_1$, for $x > 0$ at $t = 0$,
3. $n = n_2$, for $x < 0$ at $t = 0$.

$t = 0$ is the time of boundary formation, and $x = 0$ at the boundary. The concentrations on both sides of the boundary are

uniform and the refractive index on the solution side may be represented as n_1, and on the solute side as n_2.

If it is assumed that $n = T(t)X(x)$, where $T(t)$ is a function of t only and $X(x)$ is a function of x only, and this expression is differentiated and substituted back in (7), the relation

$$n_x = n_2 - \frac{n_2 - n_1}{2\sqrt{\pi D t}} \int_{\frac{-x}{2\sqrt{Dt}}}^{\left(\frac{x}{2\sqrt{Dt}}\right)} e^{-\eta^2} d\eta,$$

based on substitution of $\sigma = \frac{n_x - n_1}{n_2 - n_1}$ into Fick's second law.

may be arrived at by continuing in the same way as was done to derive Fick's diffusion equation from his second law.

It is evident that $\frac{dn}{dx} = \frac{dn}{d\eta} \frac{d\eta}{dx}$, and if $\eta = \frac{-x}{2\sqrt{Dt}}$, then

$$\frac{dn}{dx} = \frac{n_2 - n_1}{2\sqrt{\pi D t}} e^{-x^2/4Dt}$$

and $$\frac{d^2n}{dx^2} = -\frac{(n_2 - n_1)x}{4\sqrt{\pi D^3 t^3}} e^{-x^2/4Dt} .$$

The best way to find the value of the diffusion constant, D, is to use the value of x which makes $\frac{dn}{dx}$ a maximum. $\frac{dn}{dx}$ is a maximum when $\frac{d^2n}{dx^2} = 0$, which is the case when $x = 0$. If $x = 0$,

$$\frac{dn}{dx} = \frac{n_2 - n_1}{2\sqrt{\pi D t}},$$

which gives on rearranging and squaring both sides

$$Dt = \frac{(n_2 - n_1)^2}{4\pi \left(\frac{dn}{dx}\right)^2} .$$

To calculate D (13) one need know the time of diffusion, t, the value of $(n_2 - n_1)$ and $\frac{dn}{dx}$. To do this $(n_2 - n_1)$ and $\frac{dn}{dx}$ must be evaluated in terms of experimental results. Therefore a new expression for D is derived from the optical system pictured in Fig. 3a.

It is assumed that light from p enters the diffusion cell at B at an angle of incidence denoted by α_2. The cell contains a medium of continuously changing refractive index so the light is continually being bent on its passage through the cell. Refractive index is given by the expression $n = \frac{\sin\alpha_2}{\sin\alpha_1}$ where α_1 is the angle of refraction as shown in the figure. α_1 is actually the angle between B and any tangent to the curve with radius r which passes through the points A and B. This may be proved for the refractive index at B. α_2 is always greater than α_1, and for small angles $\frac{\sin\alpha_2}{\sin\alpha_1} = \frac{\alpha_2}{\alpha_1} = n_1$.

It can be proved that $r = n/\frac{dn}{dx}$ as follows: If $\frac{AB}{A'B'}$ $= \frac{\text{velocity of light at A}}{\text{velocity of light at A'}}$, and in Fig. 3b A'C is drawn parallel to B'b, $\triangle$ ABM $\sim$ $\triangle$ ACA' because the three angles are equal. Call AA' dx, then the change in the velocity of light between A and A' is AC which equals dc. Then AM = BM = r, and $\frac{AA'}{AM} = \frac{AC}{AB}$, (similar triangles), or $\frac{dx}{r} = \frac{dc}{c}$.

Refractive index is defined as $n = c_o/c$. n is the refractive index of the medium, c_o is the velocity of light in air, and c is the velocity of light in the medium. Consequently $nc = c_o$, which gives on differentiation $ndc + cdn = 0$ or $ndc = -cdn$, and $\frac{dc}{c} = -\frac{dn}{n}$. Only the absolute values are necessary, so $\frac{dc}{c} = \frac{dn}{n}$, and a substitution may be made giving $\frac{dn}{n} = \frac{dx}{r}$, so that $r = n/\frac{dn}{dx}$.

Light leaving the cell at A appears to have come from S, so that the displacement $Z = SP = b\alpha_2$. In addition, b is the optical distance from D to S (the same as the physical distance from D' to S when D' is the intersection of the straight line BP with AS). That $Z = SP = b$ is proved as follows: If x represents the distance D'C, α_2 the angle between PBD' and the line FB, and α_3 the angle between AB and AC, then $\frac{BC}{x} = \tan\alpha_2$; $\frac{BC}{\delta} = \tan\alpha_3$, so that $\delta\tan\alpha_3 = x\tan\alpha_2$ and $x = \delta\frac{\tan\alpha_3}{\tan\alpha_2}$. α_3 is the complement of angle MAB (one of the equal angles of the isosceles triangle MAB) so that $\alpha_3 = \pi/2 - \frac{1}{2}(\pi - \alpha_1)$ and

$$\tan\alpha_3 = \tan\left[\pi/2 - \frac{1}{2}(\pi - \alpha_1)\right]$$

$$= \cot\frac{1}{2}(\pi - \alpha_1)$$

$$= \frac{\sin(\pi - \alpha_1)}{1 - \cos(\pi - \alpha_1)}$$

$$= \frac{\sin\alpha_1}{1 + \cos\alpha_1}$$

Therefore $x = \frac{\delta\sin\alpha_1}{1 + \cos\alpha_1} \cdot \frac{1}{\tan\alpha_2}$

For these small angles $\tan\alpha_2 = \sin\alpha_2$, and $\cos\alpha_2 = 1$. Therefore $x = \frac{\delta\sin\alpha_1}{2\sin\alpha_2} = \frac{\delta}{2}\cdot\frac{1}{n}$, that is, one half the optical distance through the cell.

It can be shown that b, the distance from the center of the cell to the scale, is $b = \frac{GS}{1} + \frac{DC}{n} = CS + \frac{\delta}{2n} = CS + x = CS + DC$. $\sin\alpha_1 = \frac{\delta}{r}$ in $\triangle FBM$ and since $\sin\alpha_2 = n\sin\alpha_1$ so that $\sin\alpha_2 = \frac{n\delta}{r}$, and therefore $\alpha_2 = \frac{n\delta}{r}$ approximately, and also $\alpha_2 = \frac{n\delta}{n}\cdot\frac{dn}{dx}$, on substituting $r\frac{dn}{dx}$ for n, or $\alpha_2 = \delta\frac{dn}{dx}$. Since $\sin\alpha_2 = \frac{Z}{b}$, $Z = b\alpha_2$ approximately and $Z = b\delta\frac{dn}{dx}$.

Define F and G as $F = y'/x$ and $G = y'/y$ (Fig. 3c) so that $F/G = y/x = 1(1 - b)$, and $F/G = dy/dx$. This makes

$$Z = b\delta dn/dx = b\delta dn/dy \cdot dy/dx = b\delta F/G \cdot dn/dy = b\delta \frac{1}{(1 - b)} \cdot \frac{dn}{dy}.$$

Rearranging gives

$$Zdy = b\delta \frac{1}{(1 - b)} dn,$$

and $$\int_{x=-\infty}^{x=+\infty} Zy = b\delta \frac{1}{(1 - b)} \int_{x=-\infty}^{x=+\infty} dn.$$

Since $\int_{x=-\infty}^{x=+\infty} Zy$ is the area under a curve it may be represented by A and then $A = b\delta \frac{1}{(1 - b)} \cdot (n_1 - n_o)$ upon integration. The diffusion equation as previously derived is

$$\frac{dn}{dx} = \frac{(n_1 - n_o)}{2\sqrt{\pi Dt}} e^{-x^2/4Dt},$$

and it has been proved that $Z = b\delta dn/dx$ so that the maximum Z corresponds to the maximum dn/dx. The condition for dn/dx to be a maximum is that $d^2n/dx^2 = 0$, and

$$\frac{d^2n}{dx^2} = \frac{-(n_1 - n_o)xe^{-x^2/4Dt}}{4\sqrt{\pi D^3 t^3}}.$$

This has zero value when x = 0, so

$$\left(\frac{dn}{dx_{max}}\right)^2 = \frac{(n_1 - n_o)^2}{4\pi Dt}.$$

Consequently; $$D = \frac{(n_1 - n_o)^2}{4\pi t \left(\frac{dn}{dx_{max}}\right)^2}.$$

It has been shown that $(n_1 - n_o) = \frac{A}{b\delta} \cdot \frac{(1 - b)}{1}$, and

$$Z_{max} = b\delta \cdot \frac{dn}{dx_{max}}, \text{ so that substituting gives}$$

$$D = \frac{A^2}{4\pi t Z^2_{max}} \cdot \left(\frac{1 - b}{1}\right)^2.$$

In an actual experiment the base line may not coincide with

$Z = 0$ because of a small constant error in putting a plate into the comparator. For this reason, H_{max} is substituted for Z_{max} and the following equation results:

$$D = \frac{A^2}{4\pi t H^2_{max}} \cdot \left(\frac{1 - b}{1}\right)^2 \qquad (8)$$

If the photographs taken of the scale with the cell in place are not one to one reproductions of the scale, the right member of the equation would have to be multiplied by a magnification factor.

D as determined from equation (8) may be used in conjunction with the sedimentation constant determined by means of the velocity ultracentrifuge to calculate molecular weight. The equation relating these two quantities to molecular weight is

$$M = \frac{RTs}{D(1 - V\rho)}$$

where M is the molecular weight, R is the gas constant (8.3136×10^7 ergs/°C/mole), T is the absolute temperature, D is the diffusion constant, V is the partial specific volume of the protein, and ρ is the density of the buffer solution (10).

Molecular weights obtained in this way compare very well with those obtained from equilibrium ultracentrifuge data. In general, the errors in molecular weight determinations made using this equation are due largely to errors in the diffusion constant. One reason for this is that a diffusion experiment usually lasts several days and the danger of protein alteration during that time becomes considerable (10).

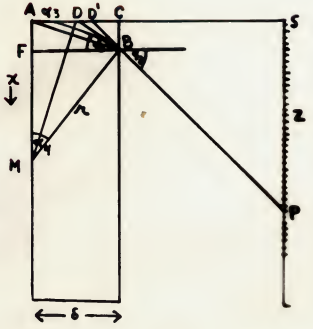
DESCRIPTION OF APPARATUS

The apparatus used for making the determination of the diffusion constant of pectinmethylesterase was the Tiselius electrophoresis assembly manufactured by Klett Manufacturing Company, New York. The modification of this apparatus used for diffusion is described by Lamm (6).

The principal features of this apparatus are a light house, a water bath, equipped with a refrigerating unit for maintaining a constant temperature, and a camera; all mounted on iron beams resting on cement supports which comprise the optical bench. (Fig. 4). The U-shaped cell, (Fig. 5) in which the diffusing substance is placed, rests in a brass rack equipped with a transparent scale. When a determination is made, the brass rack and cell are immersed in the constant temperature bath.

The transparent linear scale was made by photographing a hand drawn linear scale reduced from abount 36 inches to 55 mm in length. The scale was divided into 200 divisions, every ten of which were numbered. Positives were taken from this master scale negative, and the glass was cut to about 15 x 65 mm. Over the emulsion side of this positive a glass plate of the same size was glued in order to protect the emulsion from water (7).

Light from the mercury vapor lamp was first passed through a Wratten No. 1, Series V haze filter, and then through a Wratten No. B-58, Series V, photostating, green-monochromator slit. This was necessary because the lenses were not found to be sufficiently color corrected, so a monochromatic beam was necessary.



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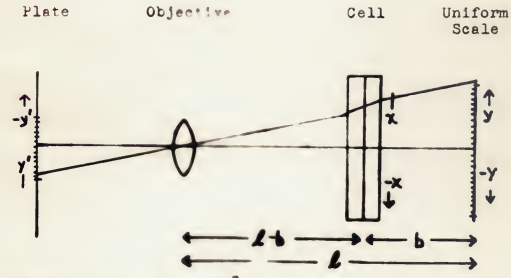

3c

Fig. 3. Light ray paths.

Light from these filters passed next through the schlieren lens on the edge of the constant temperature bath, through the transparent scale, and the boundary between the diffusing substance and the buffer, and was focused on the objective lens of the camera. This produces an inverted reversed image of the scale on the photographic plate.

Photographs of the linear scale were put into a comparator consisting of a travelling microscope securely attached to a Dietert densitometer for the purpose of accurately measuring the distance between scale lines. The travelling-microscope-densitometer comparator was found to be a convenient arrangement since the magnification produced by the densitometer made accurate reading comparatively simple and easy.

EXPERIMENTAL PROCEDURE

Preparation of Pectinmethylesterase

The enzyme was prepared as described by McColloch and Kertesz (4).

Half a bushel of frozen tomatoes from the Kansas 1947 fall crop was allowed to thaw, and then chopped to a pulp with an electric mixer. Enough salt to make a 10 percent solution was added to the pulp and the mixture was stirred for 15 minutes to insure uniform distribution of the salt. A crystal of thymol was added as a preservative, and the mixture was allowed to stand overnight in the cold room at 40°C. in a galvanized iron bucket.

The following day the pulp was strained through a finely woven cloth and skin and seeds were discarded. The juice was placed inside viscose sausage casings, each containing a thymol crystal, and these were immersed in two-liter glass bottles filled with distilled water. The distilled water was replaced every day until the liquid in the sausage casings gave a negative test for chloride ion. This dialysis required about a week.

PM precipitates in distilled water, so the dialyzate was centrifuged, and the supernatant liquid discarded. The precipitated enzyme was dissolved in 200 ml of 10 percent NaCl, and extracted for 24 hours. Part of the paste precipitated by distilled water failed to dissolve in the sodium chloride, so the 200 ml were centrifuged, and the precipitate discarded. The supernatant liquid was once more dialyzed against distilled water and centrifuged. The residue was dissolved in 200 ml of 0.5 M sodium acetate solution at pH 7.0.

Determining Enzyme Activity

The activity of enzyme preparations was determined using the method developed by Kertesz (5).

To 50 cc of 0.5 percent salt free pectin solution one cc of saturated sodium chloride was added. This solution was titrated with 0.1 N NaOH until the added Hinton indicator turned barely pink. This occurs at a pH of 6.2.

Next two cc of the enzyme solution to be tested were added to the pectin mixture, and enough NaOH was added from a burette

from time to time to keep the enzyme-pectin mixture faint pink. At the end of five minutes the number of cc of NaOH added was measured and the enzyme activity calculated from the following relation.

$$\frac{(\text{cc NaOH})(3)(30)}{(\text{time in minutes})(2)} = \text{PMU (pectinmethylesterase units)}$$

Making a Determination

In preparing an enzyme sample for use in a determination, five milliliters of the enzyme stock solution of known activity were placed inside a viscose sausage casing with a crystal of thymol, and the casing put into one liter of buffer. Dialysis was allowed to continue for one or two days. Then the enzyme was allowed to dialyze for one more day against a fresh liter of buffer. The buffer solutions were kept at 40°F., as was the stock solution.

The buffer solutions used were of two types. Some determinations were made using 0.5 M sodium acetate and some were made using veronal buffers. The preparation of veronal buffers was first described by Michaelis (8). The solution used in this work was prepared by dissolving 9.714 g of sodium acetate containing three molecules of water of hydration, 14.714 g of sodium veronal and 17.000 g of sodium chloride in sufficient distilled water, and adding distilled water to the mixture in a volumetric flask until the volume equalled 2.5 l (9). The pH was then adjusted to the desired value by the addition of

sufficient concentrated hydrochloric acid. The amounts of acid required to reach a given pH were found to vary considerably with each solution prepared. It was found impossible to use a more dilute buffer solution than this, because boundaries formed in more dilute buffer were invariably destroyed by enzyme floating upward through them.

The refrigerating unit was turned on well in advance of the time of the determination in order for the water in the constant temperature bath to reach 10° C., the temperature at which all runs were made.

In setting up the cell, the first step was to clean and dry the cell sections thoroughly with acetone. The bottom plate was next attached to the section above it with vaseline, and the bottom plate filled with the enzyme solution which had been dialyzed against two buffer solutions as described above. The filling was done by means of a hypodermic syringe with a six-inch needle. With the long needle one could fill a cell section without introducing bubbles.

After the bottom section was filled it was slid to one side so that the enzyme in it was cut off from the solution in the rest of the cell. The upper cell sections were rinsed three times with the buffer against which the enzyme had last been dialyzed, then filled with this buffer. The cell was fastened securely into the brass rack, which had the transparent scale mounted on it, and connected by means of rubber sleeves to the electrode vessels. The brass rack was set into the water bath,

and the electrode vessels were joined with a rubber siphon connection which has a three way glass stopcock on the left hand side. A connection lead from this stopcock to the compensator syringe mounted on the wall of the constant temperature bath. By means of this syringe, buffer was drawn through the siphon until it was filled, and the water levels in each electrode vessel were allowed to equalize.

In each determination the assembly stood for at least an hour in the water bath to allow temperature equilibrium to be attained. At the end of this time the bottom cell section was moved into line with the upper cell sections, and the time recorded. This time was t = 0 for the diffusion experiment.

About 10 minutes were required to move the boundary into view. After being moved into view, in the cell section just above the lower plate, the next section above was pushed out of line to assure free diffusion taking place. A photograph was taken as soon as the boundary was in view, and at 30 minute intervals thereafter.

CALCULATION OF D

Since the scale is distorted by changes in refractive index, the distorted image may be used as a means of determining the diffusion constant. The reference scale, as it is called, consists of a photograph of the scale made with the cell removed from the bath. This photograph repeats the uniform spacing of the linear scale. When a diffusion experiment is being carried

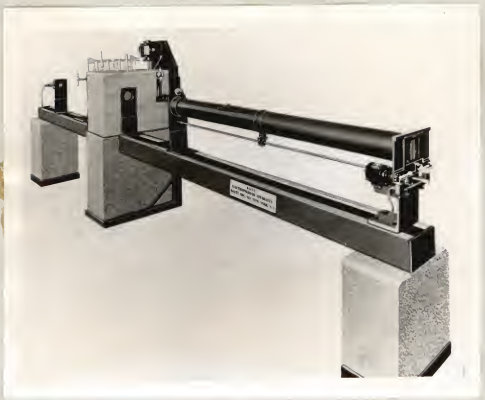

Fig. 4. The electrophoresis assembly.

Fig. 5. The electrophoresis cell.

out photographs are made, with the cell in position, at various time intervals after the formation of the boundary.

The distances between scale lines in the reference scale photograph are measured using the comparator. Photographs taken at various times during a diffusion experiment were read in a similar manner, and these values were called deviated scale readings. The readings obtained from the reference scale were subtracted from the readings of the deviated scale, and these differences (Z values) are plotted as ordinates against the deviated scale readings as abscissae. If the data are accurate, the result is a probability or normal error curve with a horizontal base line. One such curve as actually obtained from experimental data is shown in Fig. 2.

The diffusion constant was calculated using the relation

$$D = \frac{A^2}{4\pi t H^2_{max}} \cdot \left(\frac{l - b}{l}\right)^2$$

where D is the diffusion constant, A is the area under the probability curve, plotted as explained above, H is the maximum ordinate of this curve, t is the time in minutes, l is the distance from the camera objective to the scale, and b is the distance from the center of the cell to the scale.

The area, A, was actually obtained from the graph by first drawing a base line, then tracing a curve through the points above the base line. The distance between the places of intersection of the curve with the base line was measured. Then six uniformly spaced ordinates running from the base line to the curve were constructed, and their lengths were measured. These

lengths were summed together and multiplied by one seventh of the length of the base line under the curve. Measurements were made on graphs in which the scale by which abscissae were plotted was one inch equals 0.4 centimeters on the actual photographic plate, and the ordinate scale was plotted so that one inch on the graph paper represented an actual distance of 1/10 centimeter. The measurements on the graph were made with a centimeter ruler and the necessary conversion factors were used when substituting these distances into the formula. In the case of Fig. 2 (not drawn to scale) the length of the base line was 5.89 cm as measured on the centimeter ruler. $\frac{5.89}{7} = .84$. The lengths of the six ordinates were: .43, 1.08, 2.02, 2.80, 1.46, and .52, and $\Sigma = 8.31$. This makes the area $.84 \times 8.31 = 6.98$ cm^2. H_{max} as measured was 3.01, l was 186 cm, and b was 5.5 cm. The time for this experiment was 35 minutes, so that

$$D = \frac{(6.98)^2}{4x3.1416x35.(3.01)^2}\left(\frac{186 - 5.5}{186}\right)^2 \cdot \frac{(.1574)^2}{60} \text{ cm}^2/\text{sec},$$

or $$D = 4.79 \times 10^{-6} \text{ cm}^2/\text{sec}.$$

The factor 0.1574 corrects the measurements made on the centimeter ruler to the actual distances on the photographic plate.

DISCUSSION

One difficulty encountered in determining D arose from the fact that the enzyme preparations used were very dilute. This made the calculations of D after long time intervals inaccurate because the probability curve obtained by plotting Z values

Table 1.

Expt. No.[1]	Buffer	pH	Time in minutes	Percent enzyme conc.	D in cm^2/sec x 10^7
II	10% NaCl	7	35	.012	47.9
			49		12.9
			69		39.4
			88		29.1
III	10%NaOAc	6.94	70	.012	12.6
IV	10%NaOAc		116	.0049	16.8
			130		24.9
			149		13.5
V	10%NaOAc	6.94	42	.0049	2.05
			65		10.9
			95		5.42
VI	Veronal	8.7	81	.0023	33.1
VII	Veronal	8.8	125	.0023	18.9
VIII	Veronal	4.26	108	.0023	18.4
			134		14.1
IX	Veronal	4.04	238	.0023	6.92
			418		4.14
			485		5.22
X	.5MNaOAc	5.12	103	.0023	7.51
XI	.5MNaOAc	4.54	131	.0023	40.2
			162		30.5
			191		30.1
			222		24.1
			378		17.1
			386		7.73
			447		7.86
			535		4.76

[1]All experiments were run at 10° C.

against deviated scale readings became flatter as the diffusion time grew longer. The time limit was reached when the curve became so flat that errors in comparator readings were comparable in magnitude to Z values.

The preparation of a more concentrated enzyme solution was impractical because this would reduce the volume too greatly. Probably this concentration difficulty was caused by using tomatoes picked green and allowed to sun-ripen.

An approximate molecular weight may be arrived at using the relation, $M = \frac{RTs}{D(1 - V\rho)}$,

if R is taken as 8.314×10^{7} ergs per degree centigrade per mole, s is assumed to be 1.9×10^{-13} sec, D is 15×10^{-7} centimeters squared per second, V is .74, and ρ is 1.0495 grams per milliliter (the density of 10 percent sodium acetate solution).

This gives a value of 13,300 for the molecular weight. This value is probably too small for the value of D used in the equation is quite likely too large, since it was obtained after a fairly short time interval had elapsed. The value of D decreases as the time grows longer.

CONCLUSIONS

It may be concluded from this study that the enzyme pectinmethylesterase is a relatively small protein molecule. The diffusion constant appears to have the value $15 \times 10^{-7} cm^2/sec$ when measured over short time intervals, and the molecular weight

is in the neighbourhood of 13,000. Further use may be made of this value of D, in conjunction with viscosity data, in determining the ratio of major to minor axes and the degree of hydration of the molecule. This would give important information about the shape of the molecule.

It is to be hoped that further work will be done with the enzyme along these lines, and especially that the actual sedimentation constant will be determined soon.

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