STUDIES ON

I. A REINVESTIGATION OF THE KINETICS OF THE UREASE CATALYZED HYDROLYSIS OF UREA

II. ATTEMPTED PURIFICATION OF THE ADRENOCORTICOTROPIC HORMONE

III. INSTRUMENTAL ADSORPTION ANALYSIS

IV. THE EFFECT OF THE DIPHENYL HYDANTOIN (DILANTIN) ON THE ETHER NARCOSIS IN THE CAT

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ABSTRACT

In an investigation of the factors operative in the urease-catalyzed hydrolysis of urea in aqueous solutions, buffered at pH 7.0 with sodium or potassium phosphate, it has been found that both of the buffer components participate in the hydrolytic reaction. The buffer anion apparently functions as an activator and the buffer cation as an inhibitor. Sodium phosphate buffer displays a greater inhibitory action in the urease-catalyzed hydrolysis of urea than does an equivalent concentration of potassium phosphate buffer. After dialysis of urease solutions the activity in the presence of organic-inorganic buffers, e.g., ethylenediamine phosphate, was found to be greater than that of the initial preparation. The order of activation of the enzyme for the investigated anions as their ethylenediamine salts is citrate > phosphate > maleate > sulphate > chloride > acetate. The activity of the enzyme increases with increased concentrations of these buffers.

Attempted purification of three samples of adrenocorticotropic hormone, RN 145, RN 146 and RN 147, resulted in the following conclusions. Paper chromatography revealed three components in RN 145, two in RN 146 with a possible third; and three in RN 147 with a possible fourth. Further work on sample RN 147 indicated five moieties by electrophoresis on paper, five with a possible sixth by

dinitrophenylation and chromatography and a separation into five components by dialysis and fractional ammonium sulphate precipitation.

The machine built for Instrumental Adsorption Analysis at the California Institute of Technology was put into operation. Successful separations of fatty acid mixtures and an isomeric cis-trans mixture were achieved. Attempted resolution of the adrenocorticotropic hormone indicated non-homogeneity.

The administration of diphenyl hydantoin (dilantin) in a dose of fifty milligrams per kilogram bodyweight was observed to potentiate the narcotic effect of ether. It caused a forty percent decrease in blood ether concentration necessary to suppress the knee jerk of the cat. This supports the thesis that the depolarizing effects of ether and diphenyl hydantoin are additive and that depolarization is an important factor in the narcotic effect of ether on central conduction.

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PART I

A REINVESTIGATION OF THE KINETICS OF THE UREASE-CATALYZED HYDROLYSIS OF UREA

Historical Résumé

The advances achieved in the field of enzymology from the 17th century on might well be traced by the progress in the understanding of one enzyme, urease. The action of this enzyme was suggested as early as 1682, when Van Helmont[1] advanced the idea that this substance was responsible for the formation of ammonia in urine by bacterial fermentation (Micrococcus ureae). The activity of this enzyme greatly influenced the controversy of Liebig and Pasteur in their expositions on the nature of fermentation. The path which urease research followed paralleled the discoveries in the other fields of enzymology, and a milestone was reached in 1926, when Sumner[2] announced his discovery of the first crystalline enzyme, urease.

Urease is found widely distributed in the plant kingdom, and a few examples in the animal kingdom have been cited. Urease activity has been found in bacteria[3], fungi[4], molds[5], larvae[6], several plant organs and tissues, especially seeds[7], and in all leguminous plants[8]. Urease activity has been found in the gastric mucosa of man[10], dogs[9,10,11], cats[10], cows and goats[9]. Weil[12] found evidence of urease activity in red blood cells of the rat, rabbit and man. Traces have been found in the pancreas , brain, thymus, and muscle[12]. Certain organs of the horseshoe crab have been found to possess urease activity[13]. All preparations from animal sources have yielded extremely

small quantities and no careful study of these preparations has been possible.

In 1876 Musculus[3] succeeded in preparing solutions of urease from bacterial sources. Miquel[14], in 1890, studied the behavior of these bacterial urease preparations under various conditions. It was not until years later that researchers were cognizant of the fact that urease was widely distributed among plants. Kokkoji[15] found urease in Cortenellus edodes in 1907, and Shibata discovered its presence in Aspergillus niger[16]. Of the seeds the soya bean and jack bean were found to be the best sources. Takeuchi and Jonone[17] were the first to discover urease in soya bean, soya hispida. In rapid succession various workers (Keisel[18], Zemplen[19], and Annett[20]) succeeded in finding urease in various amounts in the seeds of higher plants (e.g. wheat, lupin). In 1918 Marshall and Mateer[21] found that jack bean, canavalia ensiformis, contains sixteen times as much urease as does the soya bean. Jack bean was stated to contain 0.15 percent urease.

Urease was immediately put to use in haematology by Marshall and Plimmer[22], in the estimation of blood urea by Skelton[23], and later in urine analysis.

Specific studies on urease action were begun soon after urease extracts had been obtained in relatively large amounts. Takeuchi[17] studied the influence of salts, acids and other compounds on the hydrolysis of urea, but the concentrations used were much too high to be of significance. Armstrong and Horton[24,25,26] studied the various effects of acids on

urease and numerous inhibitors were also found. Marshall[27] recorded the dependence upon pH and Van Slyke and Cullen[28] tried to purify urease to eliminate as many variables as possible. Van Slyke and co-workers[29,30] extensively studied the effect of pH and obtained values for maximum activity. Van Slyke first introduced a kinetic formula for the urease system, $t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right)$. Michaelis and Menten[31] brought forward their kinetic theory of enzyme action in 1913, which although a more general formulation basically agreed with the Van Slyke treatment when applied to urease.

The effects of various buffer systems on the urease-urea complex have been studied by Sumner and Howell[32]. Their work showed that the activity optimum depends on the type of buffer employed, and optimum pH's were obtained in the range 6.4 to 7.6 for the various systems studied. Niemann and Harmon[33] applied Michaelis kinetics and evaluated K_S values in phosphate buffers. Their studies led to the belief that phosphate was a competitive inhibitor. Further work of Niemann[34] showed that the specific activity of urease was dependent on the apparent absolute enzyme concentration. Inhibition by a product of hydrolysis, the ammonium ion, was shown by Laidler[35,36] to be of a non-competitive nature. Competitive inhibition by HSO_3^- ion and other sulfur compounds was claimed by Kistiakowsky[37,38]. Reversible inhibition by attachment of silver ion to the active site in a 1:1 ratio was reported[39]. This work

indicated three to four active sites per molecule. Inhibition by surface active agents[40]; by acetylated sulfonamides[41]; by antibiotics[42] and by diamines[43], has been investigated.

As early as 1915 the question was raised as to the possibility of a co-enzyme existing for urease. Onodera[44] described dialysis experiments in which dialyzed urease lost activity. This activity could be restored completely by the addition of a small amount of fresh urease. Kato[45] brought forward evidence which he interpreted as also suggesting a two component system. Lovgren[46] claimed that these observed effects were due to improper control of pH and later work of Sumner[47] appeared to substantiate Lovgren's claim.

Excellent reviews up to 1927 by Lovgren[46] and Oppenheimer[1] may be found.

The end product of the urease hydrolysis of urea is known to consist of ammonia and carbon dioxide. The mechanism of reaction leading to this final product has, however, given rise to much discussion. Dumas[49] in 1830 suggested the following equation:

$$\begin{matrix} NH_2 \\ C{=}O \\ NH_2 \end{matrix} + 2\ H_2O \longrightarrow (NH_4)_2CO_3$$

In 1885 Fenton[50] postulated that ammonium carbamate was an intermediate in the hydrolysis. Armstrong and Horton[25], in 1912 considered the hydrated urea molecule as entering into

the mechanism in the following form;

$$C\overset{(OH)_2}{\underset{(NH_2)_2}{=}} + 2H_2O \longrightarrow C\equiv(OH)_4 + 2NH_3$$

Yamasaki[51], in 1920, found carbamate present in the reaction mixture. Fearon[52] introduced the idea of the conversion of urea to ammonium cyanate, and the spontaneous hydrolysis of this into the final product, ammonium carbonate. Sumner[53] challenged this view; he showed that in the absence of buffers no cyanate is formed and confirmed Yamasaki's work. Laidler[35,54] has postulated a two site mechanism with the complex urea-urease-H_2O being formed as a necessary condition for hydrolysis.

Urease specificity is claimed to be absolute[56]. However Werner[57] states that mono-butyl urea is decomposed by urease. Pin Yin Yi[58] claims that Robinia urease also attacks asymmetrical dimethyl- and diethyl urea. Takeuchi first reported the hydrolysis of biuret and recently Kistiakowsky[59] reported the same result but retracted this statement when he found small amounts of urea present in the biuret[60].

Sumner and Poland[61] first reported the presence of sulfhydryl groups in the urease molecule. Hellerman[62] demonstrated that urease activity is connected with these groups. Desnuelli[63] has divided these sulfhydryl groups into three classes, one of which is highly specific for urease activity. An effect associated with the oxidation-

reduction potential of the medium was claimed by Sizer and Tytell[64]. Activity was found in the range E_h = 100 to 200 mv. with the maximum value at 150 mv., using various oxidation-reduction mixtures. Sizer[65] evaluated activation energies for urease and found two values, 11,700 cal. and 8,700 cal. depending on the E_h of the medium. Kistiakowsky refuted the idea of two values for the activation energy and the sharp break in the Arrhenius plots, and pointed out that these were due to inhibitory action[66]. Hofstee[67] studied the effect of temperature on activity and reported finding that a urease solution kept at 60° for five minutes reached a maximum activity. Urease solutions withstood freezing and when treated as above were found once again to achieve their previous activity. The temperature coefficient, Q_{10}, for the range 30°-40° was found to be 3 by Van Slyke and Cullen[28].

The urease molecule has been described as a globulin[61] with an isoelectric point of 5.0[69]. The molecular weight of crystalline urease was found to be 483,000[68]. Elementary analysis showed a composition for twice recrystallized urease to be: C = 51.6%, H = 7.1%, N = 16.0%, S = 1.2%[70]. Denaturation is readily caused by heavy metals including mercury, silver, copper, cadmium, lead, and gold[71,72]. Other inactivators include fluorides, halogens, borates, quinones, formaldehyde, and hydrogen peroxide[56]. Many protectors and activators have been used in urease studies. Two per cent neutralized gum arabic solution acts well, as

do many proteins, hydrophilic colloids, hydrocyanic acid, amino acids,and hydrogen sulfide. Inactivation of the protein has been observed to take place reversibly or irreversibly, depending on the conditions and mode of treatment. Trypsin had no effect on urease[73] while both pepsin and papain[74] destroyed its activity. Anti-urease can be produced by parenteral injection of urease into the rabbit[75].

The main role of urease in nature, as elucidated to date, is found to be in the nitrogen cycle. Bacteria hydrolyse urea to ammonia, which in turn is converted by soil bacteria to nitrites and nitrates. These latter products go into the protein structure of plants which are eaten by animals. The digestive waste products of such animals contain large amounts of urea, which is secreted to re-enter the cycle once more. The exact function of urease in the plant is unknown, but speculation has led to the belief that its function is to prevent the accumulation of large amounts of urea in the plant. The role of urease in the gastric mucosa of animals has not been clarified. Fitzgerald[10] and Franklin[76] postulated that urease activity was correlated with the pH of the stomach. Glick[77] concluded that urease activity is not directly involved in acid production and found evidence that urease, by releasing ammonia, has a role in regulating gastric acidity and in protecting the mucosa against the action of pepsin.

This brief review of significant facts in urease enzymology has been presented as a basis for the criticism and experimental work to be presented in the following pages.

PART I

Section 1

General Considerations of Enzyme Kinetics

and

Postulated New Mechanisms

Section 1

When kinetic behaviors were observed in urease studies (Section 2) which did not fall within any existing theories a re-examination of enzyme kinetics was necessitated. Therefore the following considerations are presented.

a. Michaelis-Menten Kinetics

The classical Michaelis-Menten[31] theory of enzyme kinetics is based on the formation of an intermediate complex in equilibrium with the enzyme and substrate.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad (1)$$

$$ES \xrightarrow{k_3} E + \text{Products} \qquad (2)$$

The assumed equilibrium is defined by

$$K_S = \frac{(E_f)(S_f)}{(ES)} = \frac{[(E) - (ES)]}{(ES)} (S_f) \qquad (3)$$

where E = total enzyme concentration, E_f = free enzyme concentration and S_f = free substrate concentration. If k_3 is the velocity constant of the decomposition of ES to give E and products and v the velocity of hydrolysis, i.e., $\frac{-(dS)}{dt}$, then

$$v = k_3 (ES)$$

and

$$v = k_3 \frac{(E)(S_f)}{K_S + (S_f)} \qquad (4)$$

For $S_f \gg K_S$, corresponding to saturation of enzyme by substrate, (4) becomes, $v = k_3(E) = V$. Finally, use of V and the

assumptions that $(S_f) \backsimeq (S)$, and $(S) \gg (ES)$, gives

$$v = \frac{V\,(S)}{K_S + (S)} \quad , \tag{5}$$

which in the reciprocal form

$$\frac{1}{v} = \left[\frac{K_S}{V}\right]\frac{1}{(S)} + \frac{1}{V} \tag{6}$$

can conveniently be handled graphically[79]. For $(S) = K_S$ the rate is half maximal, i.e., equal to $\frac{1}{2}V$.

As was pointed out by Haldane[78], this treatment assumes an equilibrium which cannot exist and fails to describe properly the steady state which may exist. Indeed the net rate of formation of ES is

$$d\,\frac{(ES)}{dt} = k_1\left[(E) - (ES)\right]\,(S) - k_2\,(ES) - k_3\,(ES),$$

so that the assumption of a steady state, with $\frac{d(ES)}{dt} = 0$, leads to the same rate expression as before with K_S equal to $\frac{k_2+k_3}{k_1}$.

Extensions of this idea of complex formation to include inhibition were made by Lineweaver and Burk[79], and by Ebersole, Guttentag, and Wilson[80], and have been summarized by Harmon and Niemann[33].

b. Type A . ES - Active Complex

Only selected equilibria were considered in the Ebersole kinetic formulation. The species involved were limited to E, S, ES, EI, and ESI, with the implication that there are at

least two different sites[1] of attachment on the enzyme molecule. However if trimolecular species are permitted, either due to two sites on the enzyme (e.g., S-E-I) or consecutive binding (e.g., E-S-I) of the entities, one must also consider the two species ESS and EII. Therefore in a system containing enzyme, E, substrate, S, and inhibitor, I, the following equilibria are maintained:

$$K_1 = \frac{(E_f)(S)}{(ES)} \quad , \quad K_2 = \frac{(E_f)(I)}{(EI)} \quad , \quad K_3 = \frac{(I)(ES)}{(ESI)} \quad ,$$

$$K_4 = \frac{(I)(EI)}{(EII)} \quad , \quad K_5 = \frac{(S)(ES)}{(ESS)}$$

The formation of products is governed by the equations,

$$ES \xrightarrow{k_3} \text{Products} + E$$

$$ESI \xrightarrow{k_4} \text{Products} + E + I$$

$$ESS \xrightarrow{k_5} \text{Products} + E + S$$

If the reaction velocity v is proportional to the concentration of the complexes containing ES, and $v = \frac{-d(S)}{dt}$, then

$$v = k_3 (ES) + k_4 (ESI) + k_5 (ESS). \qquad (7)$$

- -

1. Since the completion of this section Laidler, K. J. and Hoare, J. P. have given a similar interpretation (Ref. 35,36,54).

When S is large enough to saturate the enzyme (i.e., limiting velocity), then $v' = k_3(E) = V'$, $v'' = k_4(E) = V''$ and $v''' = k_5(E) = V'''$. Solving for the above v's the following are obtained:

$$v' = \frac{V'(S)}{K_1 + (S) + \frac{K_1}{K_2}(I) + \frac{(S)(I)}{K_3} + \frac{(I)^2 K_1}{K_2 K_4} + \frac{(S)^2}{K_5}} \quad (8)$$

$$v'' = \frac{1}{K_3}\left[\frac{V''(S)(I)}{K_1 + (S) + \frac{K_1}{K_2}(I) + \frac{(S)(I)}{K_3} + \frac{(I)^2 K_1}{K_2 K_4} + \frac{(S)^2}{K_5}}\right] \quad (9)$$

$$v''' = \frac{1}{K_5}\left[\frac{V'''(S)^2}{K_1 + (S) + \frac{K_1}{K_2}(I) + \frac{(S)(I)}{K_3} + \frac{(I)^2 K_1}{K_2 K_4} + \frac{(S)^2}{K_5}}\right] \quad (10)$$

and $v = v' + v'' + v'''$

This formulation involves two species, EII and ESS, not previously discussed. The latter can be defined as a species causing inhibition of ES due to high substrate concentration when K_5 is relatively small and $k_5 < k_3$.

Assuming k_4 and k_5 are equal to zero the various types of inhibition cited in the literature are obtained from the reciprocal of equation (8).

$$\frac{1}{v} = \left[\frac{K_1}{V}\right]\frac{1}{(S)}\left[1 + \frac{(I)}{K_2} + \frac{(I)^2}{K_2 K_4}\right] + \frac{1}{V}\left[1 + \frac{(I)}{K_3} + \frac{(S)}{K_5}\right] \quad (12)$$

In the absence of inhibitor ($I = 0$) and $K_5 = \infty$, equation (12) reduces to the Michaelis-Menten equation (4). The equation for competitive inhibition (80) is obtained by assuming K_3, K_4, and K_5 equal to ∞. Non-competitive inhibition is obtained

by setting K_4 and K_5 equal to ∞ and uncompetitive inhibition by allowing K_2, K_4, and K_5 to equal ∞ .

These simplified velocity expressions yield straight lines when $\frac{1}{v}$ versus $(\frac{1}{S})$ is plotted.

c. Type B. ESA Active Complex

In the above mechanisms the ES complexes or their activated forms are the entities which enter into final hydrolysis. If it is assumed that these complexes must be activated either by means of a collision with a third molecule or by the addition of an activator the following equilibria have to be considered for a system containing E, S, I, and A, the activator:

$$K_1 = \frac{(E_f)(S)}{(ES)} \quad , \quad K_2 = \frac{(E_f)(I)}{(EI)} \quad , K_3 = \frac{(I)(ES)}{(ESI)} \quad , \quad K_4 = \frac{(I)(EI)}{(EII)}$$

$$K_5 = \frac{(S)(ES)}{(ESS)} \quad , \quad K_6 = \frac{(A)(ES)}{(ESA)} \quad , \quad K_7 = \frac{(A)(EI)}{(EIA)} \quad , \quad K_8 = \frac{(E_f)(A)}{(EA)}$$

$$K_9 = \frac{(ESA)(I)}{(ESAI)}$$

The equations governing the formation of products are,

$$ESA \xrightarrow{k_6} \text{Products} + E + A$$

$$ESAI \xrightarrow{k_7} \text{Products} + E + A + I$$

The velocity is proportional to the concentration of the complexes containing ESA,

$$v = k_6 (ESA) + k_7 (ESAI). \qquad (13)$$

Let $v' = k_6(E) = V'$ and $v'' = k_7(E) = V''$. Solving for the above, the following equations are obtained:

$$v' = V'(S)(A)/\left[K_1K_6 + (A)(S) + K_6(S) + \frac{(A)(S)(I)}{K_9} + \frac{K_1K_6(I)}{K_2} + \frac{K_6(I)(S)}{K_3}\right.$$

$$\left. + \frac{K_6(S)^2}{K_5} + \frac{K_1K_6(A)}{K_8} + \frac{K_1K_6(I)^2}{K_2K_4} + \frac{K_1K_6(A)(I)}{K_2K_7}\right] \qquad (14)$$

$$v'' = V''(S)(A)(I)/\ K_9\left[K_1K_6 + (A)(S) + K_6(S) + \frac{(A)(S)(I)}{K_9} + \frac{K_1K_6(I)}{K_2}\right.$$

$$\left. + \frac{K_6(I)(S)}{K_3} + \frac{K_6(S)}{K_5} + \frac{K_1K_6(A)}{K_8} + \frac{K_1K_6(I)^2}{K_2K_4} + \frac{K_1K_6(A)(I)}{K_2K_7}\right] \qquad (15)$$

and $$v = v' + v''. \qquad (16)$$

These equations give rise to straight line plots of $\frac{1}{v}$ versus $\frac{1}{(S)}$ at constant activator and inhibitor concentrations and $K_5 = \infty$. However, as will be shown later it is necessary to define systems in which although the activator and inhibitor concentrations are kept constant, the plotting of $\frac{1}{v}$ versus $\frac{1}{(S)}$ gives rise to curves. The mechanisms given below lead to this condition.

d. Type C - ESS active Complex

Both substrate and inhibitor may attack both sites on the enzyme molecule in this mechanism. Furthermore let a necessary condition for hydrolysis be that two molecules of

substrate be attached to the enzyme molecule. In a system containing E, S and I the following equilibria are maintained:

$$K_1 = \frac{(E_f)(S)}{(ES)} , \quad K_2 = \frac{(E_f)(I)}{(EI)} , \quad K_3 = \frac{(I)(ES)}{(ESI)} , \quad K_4 = \frac{(I)(EI)}{(EII)}$$

$$K_5 = \frac{(S)(ES)}{(ESS)} , \quad K_{10} = \frac{(S)(ESS)}{(ESSS)} , \quad K_{11} = \frac{(I)(ESS)}{(ESSI)} .$$

The equations governing the formation of products are,

$$ESS \xrightarrow{k_8} \text{Products} + E$$

$$ESSS \xrightarrow{k_9} \text{Products} + E + S$$

$$ESSI \xrightarrow{k_{10}} \text{Products} + E + I$$

The velocity expression is,

$$v = k_8(ESS) + k_9(ESSS) + k_{10}(ESSI) \qquad (17)$$

Both ESSI and ESSS represent species which cause inhibition due to inhibitor and excess substrate if $k_9 < k_8$ and $k_{10} < k_8$. Let $v' = k_8(E) = V'$, $v'' = k_9(E) = V''$ and $v''' = k_{10}(E) = V'''$. Solving for these above 4 equations, the following are obtained:

$$v' = \frac{V'(S)^2}{K_1K_5 + (S)^2 + K_5(S) + \frac{(S)^3}{K_{10}} + \frac{(S)^2(I)}{K_{11}} + \frac{K_1K_5(I)}{K_2} + \frac{K_5(S)(I)}{K_3} + \frac{K_1K_5(I)^2}{K_2K_4}} \qquad (18)$$

$$v'' = \frac{V''(S)^3}{K_{10}\left[K_1K_5 + (S)^2 + K_5(S) + \frac{(S)^3}{K_{10}} + \frac{(S)^2(I)}{K_{11}} + \frac{K_1K_5(I)}{K_2} + \frac{K_5(S)(I)}{K_3} + \frac{K_1K_5(I)^2}{K_2K_4}\right]} \qquad (19)$$

$$v''' = V'''(S)^2(I) / K_{11}\left[K_1K_5 + (S)^2 + K_5(S) + \frac{(S)^3}{K_{10}} + \frac{(S)^2(I)}{K_{11}} + \frac{K_1K_5(I)}{K_2}\right.$$

$$\left. + \frac{K_5(S)(I)}{K_3} + \frac{K_1K_5(I)^2}{K_2K_4}\right] \qquad (20)$$

and $$v = v' + v'' + v'''. \qquad (21)$$

The plot derived from $\frac{1}{v}$ versus $\frac{1}{(S)}$ is a curve at constant inhibitor concentration.

e. Type D - ESSA - Active Complex

The ESS mechanism can be extended to include the condition of having an activator present. Thus in a system containing E, S, I, and A, with the active complex ESSA, the following equilibria are maintained:

$$K_1 = \frac{(E_f)(S)}{(ES)}, \quad K_2 = \frac{(E_f)(I)}{(ES)}, \quad K_3 = \frac{(I)(ES)}{(ESI)}, \quad K_4 = \frac{(I)(EI)}{(EII)},$$

$$K_5 = \frac{(S)(ES)}{(ESS)}, \quad K_6 = \frac{(A)(ES)}{(ESA)}, \quad K_7 = \frac{(A)(EI)}{(EIA)}, \quad K_8 = \frac{(E_f)(A)}{(EA)},$$

$$K_9 = \frac{(I)(ESA)}{(ESAI)}, \quad K_{10} = \frac{(S)(ESS)}{(ESSS)}, \quad K_{11} = \frac{(I)(ESS)}{(ESSI)}, \quad K_{12} = \frac{(A)(ESS)}{(ESSA)}.$$

The rate equation is $v = k_9(ESSA)$ (22)

Let $v = k_9(E) = V$, then

$$\frac{1}{v} = \left[\frac{K_1K_5K_{12}}{V}\right]\frac{1}{(S)^2(A)}\left[1 + \frac{(I)}{K_2} + \frac{(I)^2}{K_2K_4}\right] + \left[\frac{K_5K_{12}}{V}\right]\frac{1}{(S)(A)}\left[1 + \frac{(I)}{K_3}\right]$$

$$+ \left[\frac{K_{12}}{V}\right]\frac{1}{(A)}\left[1 + \frac{(I)}{K_{11}} + \frac{(S)}{K_{10}}\right] + \left[\frac{K_1K_5K_{12}}{V}\right]\frac{1}{(S)^2}\left[\frac{1}{K_8} + \frac{(I)}{K_2K_7}\right]$$

$$+ \left[\frac{K_5K_{12}}{V}\right]\frac{1}{S}\left[1 + \frac{(I)}{K_4K_6}\right] + \frac{1}{V} \qquad (23)$$

At constant activator concentration the curve obtained by plotting $\frac{1}{v}$ versus $\frac{1}{(S)}$ is a curve similar to that obtained for the ESS active complex mechanism.

The last example points out the fact that it is possible to obtain similar curves of v or $\frac{1}{v}$ versus (S) or $\frac{1}{(S)}$, from completely different mechanisms. Other examples can be seen from the following:

In the system where activation is necessary, Type B active complex ESA, with no inhibitor present and assuming $K_5 = \infty$, the velocity expression becomes,

$$\frac{1}{v} = \left[\frac{K_1K_6}{V}\right] \frac{1}{(S)(A)} + \left[\frac{K_6}{V}\right] \left[\frac{1}{(A)} + \frac{1}{K_8(S)}\right] + \frac{1}{V} \quad (24)$$

A plot of $\frac{1}{v}$ versus $\frac{1}{(S)}$ would produce a curved line. However if $(A) = \alpha\ (S)$ where $\alpha \simeq 1$ the expression, (15), becomes,

$$\frac{1}{v} = \left[\frac{K_1K_6}{V}\right] \frac{1}{\alpha(S)^2} + \left[\frac{K_6}{V}\right] \frac{1}{\alpha(S)} \left[1 + \frac{1}{K_8}\right] + \frac{1}{V} \quad (25)$$

which has exactly the same form as the reciprocal of equation (20), where the active complex is ESS, with the conditions that $I = 0$ and $K_{10} = \infty$. A similar equation is obtained when the active species is ESSA and the activator concentration is constant.

Summary

Three additional mechanisms have been postulated for enzyme reactions. In the first an activator is either a part of the active complex or must collide with it. Usually this does not involve any change in the interpretation of the curves obtained by plotting $\frac{1}{v}$ versus $\frac{1}{(S)}$ at a constant activator concentration.

In the second, the active complex is ESS and at either of the two enzyme sites S may be replaced by I. The plots yield curves.

In the third an activator is required in addition to the two substrate molecules, the active complex being SESA.

Caution is advised in interpreting curves as widely different mechanisms can lead to similar plots.

Part 1

Section 2

THE ACTIVITY OF UREASE IN THE PRESENCE OF SODIUM AND POTASSIUM PHOSPHATE

a. Résumé of Experimental Results

At 25° and pH 7.0 the urease catalyzed hydrolysis of urea was found to be:

1. Accelerated by an initial increase in the buffer concentration in each of the buffer systems sodium phosphate, potassium phosphate and potassium maleate,

2. Inhibited by further increase in the concentration of sodium phosphate and potassium phosphate (maleate not studied), sodium phosphate effecting the greater inhibition, and

3. Inhibited by the neutral salts sodium chloride and potassium chloride.

The potassium phosphate system obeys Michaelis-Menten kinetics, but the sodium phosphate system does not.

b. Discussion of Results

Enzyme systems are usually studied at their pH optima, in buffered solutions. But for urease, it has been reported[32] that the pH optimum is not the same for all buffers and that phosphate is an inhibitor[30,32,81]; Harmon and Niemann[33], moreover, remarked that ionic strength may also be an important variable. The present work shows, apparently for the first time, that different buffer cations also have different effects on the activity of urease.

At 25° and pH 7.0 in sodium or potassium phosphate buffer, in addition to their action as buffers, the phosphate ion apparently functions as an activator and the sodium or potassium as an inhibitor.

Though the phosphate anion has been stated to be a competitive inhibitor[33], it will be assumed in this thesis that this inhibition was due to the same effect observed in the present experiments, namely due to the inhibition by the cation. That acceleration by the phosphate anion is strongly indicated will be shown to be true in Part 1, Section 3.

The possibility that these apparent activators and inhibitors act by combining with indigenous activators and inhibitors cannot be overlooked. Thus if the phosphate ion complexed with an indigenous inhibitor the resulting effect would be an observed activation.

Relative differences in inhibition between the cations, sodium ion and potassium ion, were also found. Differences due to anions have been recorded previously[32]. From the data given in Figures I and II (Tables 1,2,3,4) and Tables 5 and 6, it can be seen that at equivalent buffer concentrations and with all other factors held constant, the activity of urease is greater in a potassium phosphate buffer than in a sodium phosphate buffer. Furthermore if sodium chloride is added to a system containing urease, urea and sodium phosphate, or potassium chloride to a system containing urease, urea and potassium phosphate, a significant dimunition in the activity of the urease is observed (cf. Figure III, Tables 6 and 7). Again the sodium salt causes greater inhibition than the potassium salt at equivalent concentrations. The effect of inhibition by these salts was observed as early as 1912 by Armstrong and Horton[25].

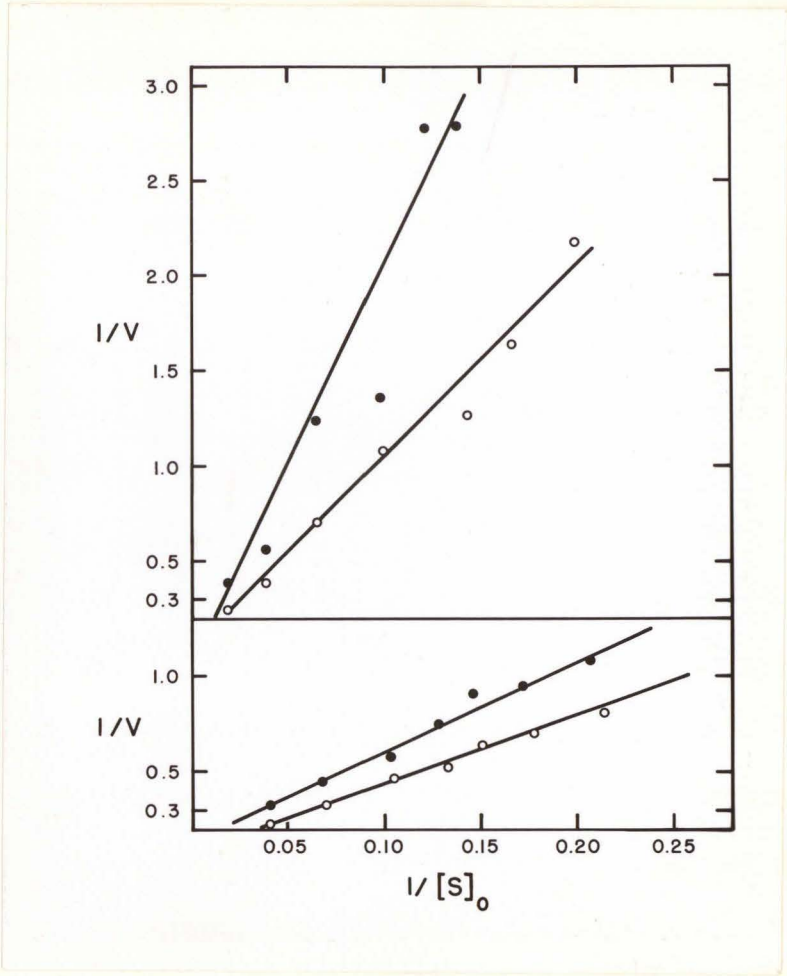

Fig. I. Activity of urease in sodium and potassium phosphate buffers; $\frac{1}{v}$ in micromoles of ammonia per ml. per min; $\frac{1}{(S)_o}$ in micromoles of urea per ml.; solid circles sodium phosphate; open circles potassium phosphate; upper plot 0.055 molar phosphate; lower plot 0.214 molar phosphate.

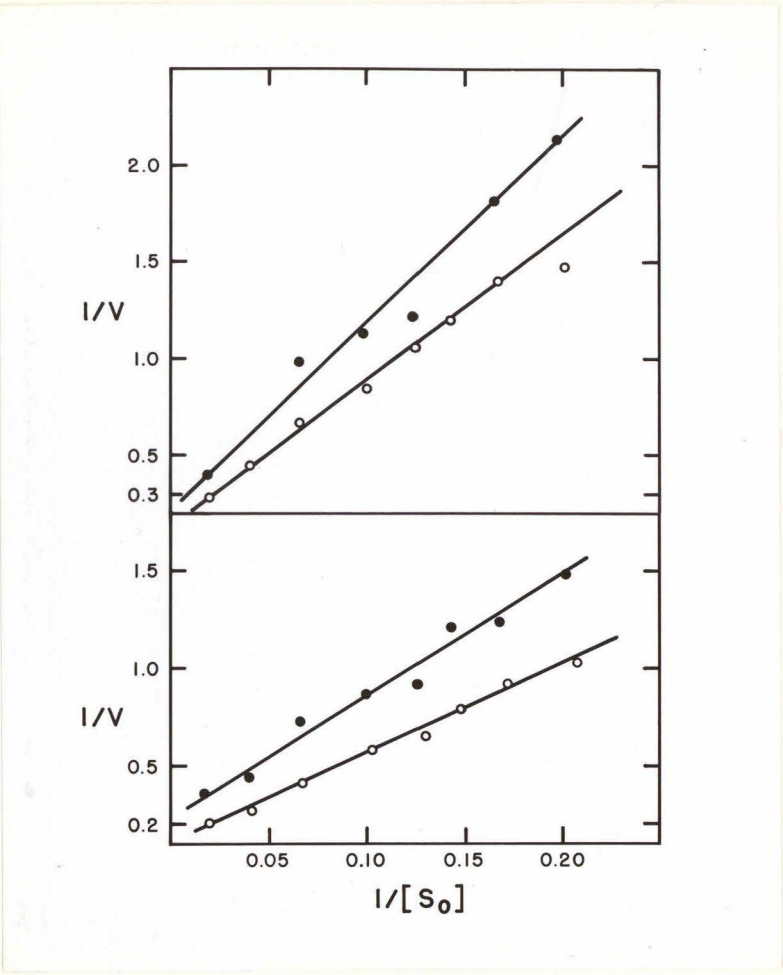

Fig. II. Activity of urease in sodium and potassium phosphate buffers; $\frac{1}{v}$ in micromoles of ammonia per ml. per min.; $\frac{1}{(S)_o}$ in micromoles of urea per ml.; solid circles sodium phosphate; open circles potassium phosphate; upper plot 0.267 molar phosphate; lower plot 0.150 molar phosphate.

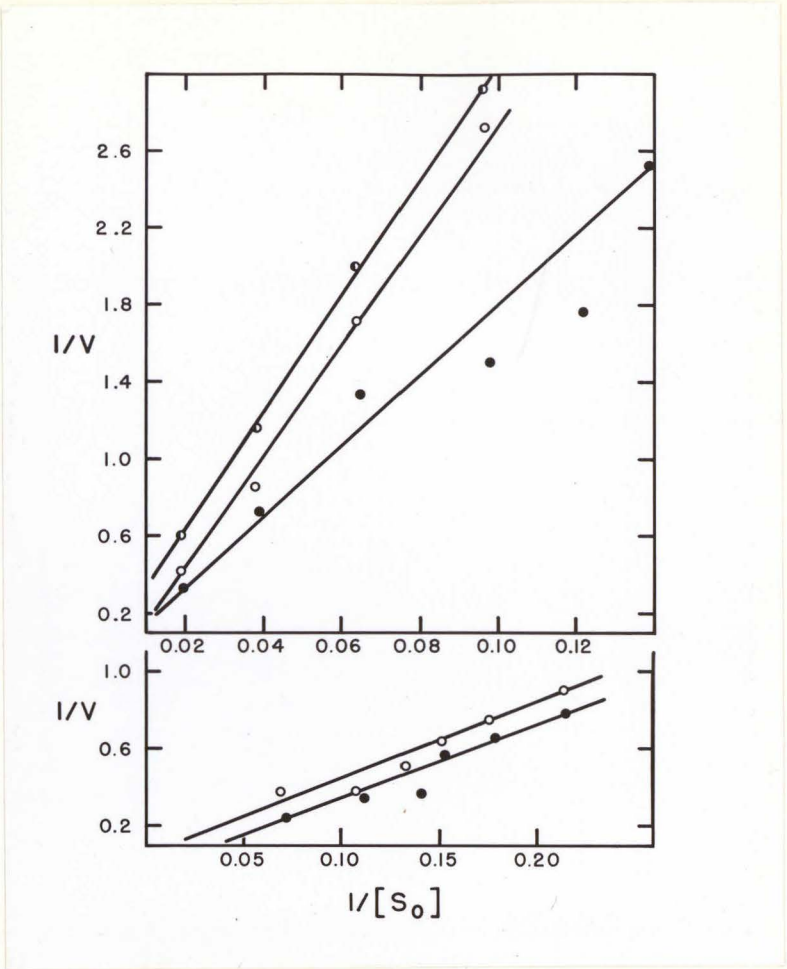

Fig. III. Effect of added sodium or potassium chloride upon the activity of urease; $\frac{1}{v}$ in micromoles of ammonia per ml. per min.; $\frac{1}{(S)_o}$ in micromoles urea per ml.; upper plot all solutions 0.05 molar in sodium phosphate buffer, solid circles no added sodium chloride, open circles 0.1 molar in added sodium chloride, half open circles 0.265 molar in added sodium chloride; lower plot all solutions 0.05 molar in potassium phosphate buffer, solid circles no added potassium chloride, open circles 0.1 molar in added potassium chloride.

Chloride ion itself, in common with a number of other anions in the form of their ethylenediamine salts, appears to activate urease weakly (See Part 1, Section 3) and must be considered in the overall effect of these neutral salts.

Concurrent inhibition and activation in buffer systems can be seen from considerations of the behavior of urease in both potassium and sodium phosphate buffers.

With potassium phosphate buffers at 25° and pH 7.0 the urease catalyzed hydrolysis of urea appears to follow Michaelis-Menten kinetics, in that for any given potassium phosphate concentration, the conventional $\frac{1}{v}$ versus $\frac{1}{(S)}$ plot was found to be linear within the limits of experimental error. However, with increasing potassium phosphate concentration the activity of urease was observed to increase and then to decrease (cf. Figure IV, Tables 9 and 10; also Tables 2,3,4,5 of series 1 and Tables 11, 12, 13, 14 of series 2). When the slope of the $\frac{1}{v}$ versus $\frac{1}{(S)}$ plot for any given potassium phosphate concentration was in turn plotted against the potassium phosphate concentration it was noted that the urease exhibited maximum activity at a potassium phosphate concentration of approximately 0.16 M for one particular set of experiments in series 1 (cf. Figure V, Table 15). A similar plot is obtained from series 2, Table 16. It is seen that the buffer of least concentration, 0.055 M, displays the greatest inhibition in all cases. Using the minimum slope value from Figure V, 4.7, at a phosphate concentration of 0.158 M, for which the intercept

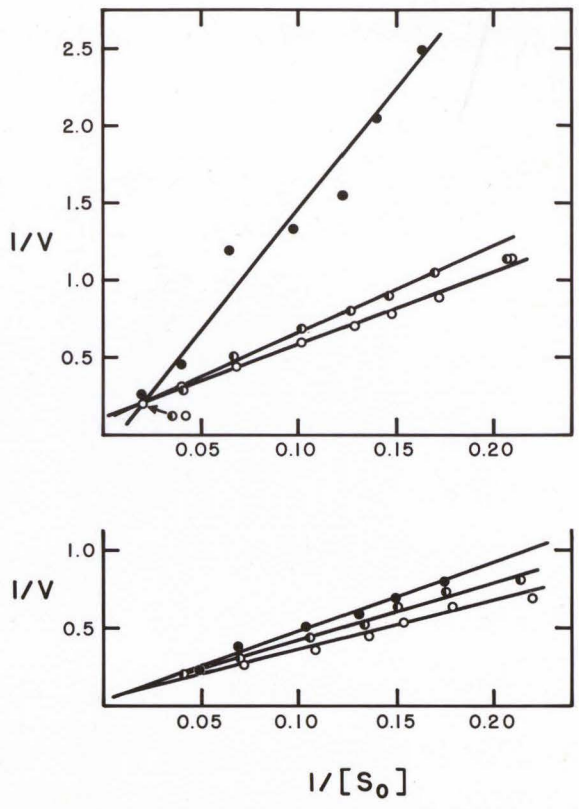

Fig. IV. Activity of urease with increasing potassium phosphate concentration; $\frac{1}{v}$ in micromoles of ammonia per ml. per min.; $\frac{1}{(S)_o}$ in micromoles of urea per ml.; upper plot, open circles 0.159 molar phosphate, half open circles 0.108 molar phosphate, solid circles 0.055 molar phosphate; lower plot, open circles 0.159 molar phosphate, half open circles 0.214 molar phosphate, solid circles 0.267 phosphate.

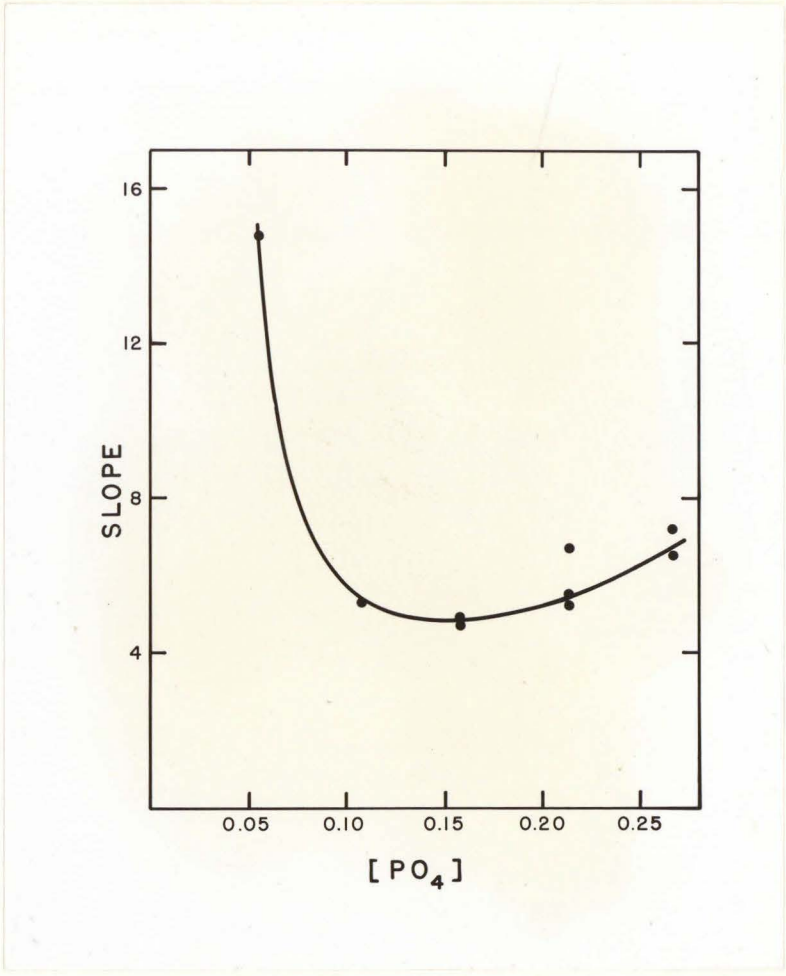

Fig. V. Slope of $\frac{1}{v}$ versus $\frac{1}{(S)_o}$ plot versus potassium phosphate buffer concentration in moles of phosphate per liter.

value of 0.144 was obtained, a K_S value of 0.03 M urea is calculated. One realizes from analysis of Figure V that for phosphate concentrations less than 0.158 M, attempted evaluation of K_S at zero phosphate concentration would produce very large values, while extrapolation for K_S using phosphate concentrations greater than 0.158 M would lead to very small values. It is possible to see how straight line plots could be obtained on extrapolation for K_S at zero phosphate concentration working on either side of the minimum. This is believed to be the reason that Harmon and Niemann(33) produced straight line plots for potassium phosphate buffers. Although the concentrations used were similar, the fact that these workers were working on the right side of the minimum (Figure V) at all times can be understood from the consideration that the relative values for the buffer concentration will be determined by the concentration of the active species of the enzyme. Although the present work was done at enzyme protein nitrogen concentrations similar to the above mentioned workers, this cannot be taken as a relative measure of the concentration of active species[1].

The fact that under the conditions used in this work K_S is a variable, using Michaelis-Menten kinetics, is seen in Table 24. The value of 0.003 M urea for K_S obtained by Harmon and Niemann(33) was found in Experiment II-5.

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1. Denaturation with time would not affect the nitrogen concentration, while the active species would be depleted.

The correlation of experiments of series one and two can be seen from Table 17. These represent experiments with different enzyme preparations, carried out over an extended period of time. Yet the ratios of the slopes for the different concentrations of potassium phosphate buffers is seen to be fairly constant.

Potassium maleate buffers displayed the same activity effect. On initial increase of buffer concentration increased urease activity was observed (cf. Tables 18 and 19).

A re-examination of the potassium maleate curves of Harmon and Niemann[33] shows that although this type of inhibition is defined as uncompetitive inhibition, the value of K_I calculated from their results does not remain constant. Using the relation $\frac{1}{v} = \frac{1}{V}\left[1 + \frac{(I)}{K_I}\right] + A$, where A equals $\left[\frac{K_S}{V}\ \frac{1}{(S)}\right]$, v is the measured velocity, V the maximum velocity and K_S the usual Michaelis-Menten constant, and values from Table 1[33], it is found that for the first two buffer concentrations the value of $K_I = (0.26 - 1.6\ A) \times 10^{-3}$ M is obtained, and for the second and third values of buffer concentrations a value of $K_I = (0.44 - 0.42\ A) \times 10^{-3}$ M. Thus K_I also changes with different buffer concentrations.

This "antagonistic" buffer effect is also observed with sodium phosphate buffers. However, with sodium phosphate buffers the $\frac{1}{v}$ versus $\frac{1}{(S)}$ plots were not linear except at low sodium phosphate concentrations (cf. Figure VI, Tables 20, 21, Tables 22, 23). Nevertheless from the data given in

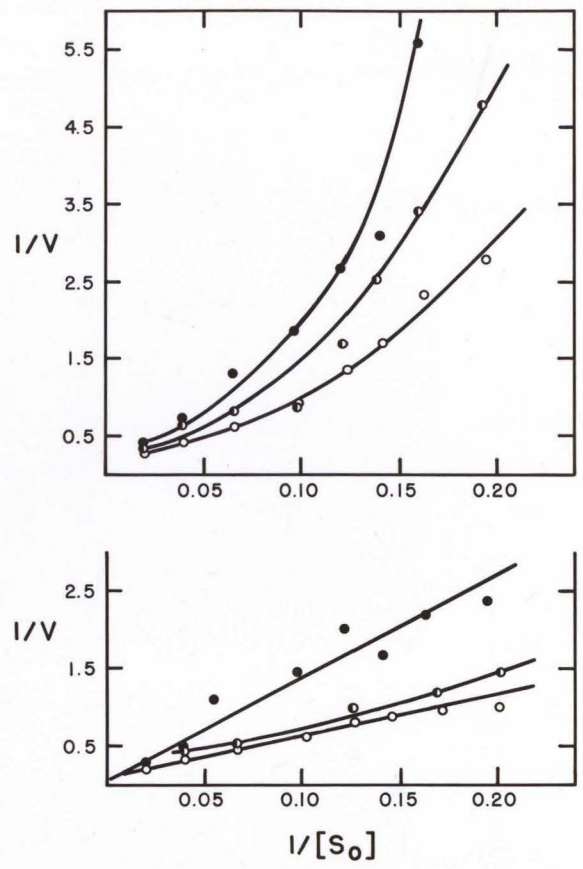

Fig. VI. Activity of urease with increasing sodium phosphate concentration; $\frac{1}{V}$ in micromoles of ammonia per ml. per min.; $\frac{1}{(S)_o}$ in micromoles of urea per ml.; upper plot, open circles 0.158 molar phosphate, half open circles 0.214 molar phosphate, solid circles 0.267 molar phosphate; lower plot, open circles 0.108 molar phosphate, half open circles 0.158 molar phosphate, solid circles 0.055 molar phosphate.

Figure VI it is clear that here also the activity of urease increased and then decreased with increasing sodium phosphate concentration. The fact that sodium phosphate buffers display greater inhibition than do potassium phosphate buffers can be seen by comparing Figures IV and VI. Increasing the potassium phosphate concentration from 0.055 M to 0.158 M causes increased activity of urease, at which point further increases in concentration of buffer decreases the activity. While for sodium phosphate, increased urease activity is observed only from 0.055 M to 0.108 M, at which point further increases of buffer concentration causes decreased activity.

Analysis of the curves of Figure VI - upper plot at high sodium phosphate concentration, and the 0.108 M curve Figure VI lower plot, reveals that these curves belong to a family of parabolas. Analysis of the curves for the two lower buffer concentrations, Figure VI lower plot, shows that these are straight lines as predicted by Michaelis-Menten kinetics. As the steepness of the curve determines the degree of inhibition, the sodium ion appears to rapidly cause greater inhibition with increasing concentration. This series of curves (Figure VI) exhibits the phenomena of slowly passing from straight line plots to parabolas.

A summary of all pertinent data is presented in Table 24.

The conclusion that sodium or potassium ion can function as an apparent inhibitor in an activated urease-urea system derives support from earlier observation on the

inhibitory action of sodium and potassium chloride in urease-urea systems containing no added buffer[25,82]. In fact E. F. and H. E. Armstrong[26], if their work with crude (rather than crystalline) urease is valid, first established that with potassium phosphate buffer the activity first increases and then decreases with increasing concentration of buffer. Also at equivalent concentration of sodium or potassium phosphate, greater activity was observed in the potassium buffer than in the sodium buffer[1].

Van Slyke and Cullen[28] observed that the extent of hydrolysis of urea, in an initially unbuffered system by a crude urease preparation, first increased and then decreased upon the addition of increasing concentrations of potassium phosphate, and these authors stated that the above effect was due to a change in the pH of the reaction system, which apparently was not measured or at least not reported. A reconsideration of the original data has shown that the above explanation cannot be correct and that the effect observed undoubtedly arose from activation by added phosphate ion and inhibition by added potassium ion. This conclusion is based on the following consideration. Reproduced are the figures from Table VII[28] as columns a and d below.

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1. See also N. Kato, Reference (45).

(a)[1] M KH_2PO_4	(b) Moles(a) x 10^{-3}	(c) 3/7 (b)	(d) 0.02N NH_3 cc's formed in 15 mins	(e) moles of(d) x 10^{-3}	(f)[2] excess (d)x10^{-3} M
0.016	0.099	0.042	24.00	0.48	0.44
0.031	0.198	0.085	24.96	0.498	0.41
0.045	0.297	0.127	24.75	0.497	0.37
0.059	0.402	0.172	20.00	0.400	0.23
0.071	0.497	0.213	14.62	0.292	0.08

The explanation given for the effect observed was based on the change of pH during the hydrolysis. However this cannot be a valid explanation as seen from column (f) above. These calculations are made on the following basis. For the optimum pH of 7.0 to be obtained, 3/7 of the $H_2PO_4^-$ would have to be converted to HPO_4^{--}. The ammonia liberated would cause this transition and column (f) represents the excess of ammonia liberated over the amount required for this transition. The pH of 0.016 M KH_2PO_4 is 4.6 and of 0.071 M, 4.1. Thus all runs started at roughly the same pH, on the acid side of the optimum pH, and ended up on the basic side of the optimum. The first run should have arrived at this alkaline pH before the others and thus operated under the most unfavorable conditions throughout the hydrolysis. Yet the first concentration, 0.016 M, of buffer produced greater hydrolysis than did the more highly buffered 0.071 M. Therefore their conclusion is invalid.

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1. Concentration of potassium phosphate in the reaction mixture.

2. (e) - (c) = excess NH_3.

Sizer[64] has noted that urease activity increased and then decreased with increasing concentrations of added sodium sulphate and Schmidt[72] found that sodium fluoride caused a greater inhibition of the urease-urea system than did potassium fluoride. Although the former author's explanation was that the observed effect was due to a change in the E_h of the medium, and the latter author's explanation was based upon an assumed inhibition by fluoride ion, it is reasonably clear that here again activation by the added anion and inhibition by the added cation is the more likely explanation.

Within the last year Kistiakowsky and Lumry[66] and Ambrose, Kistiakowsky and Kridle[38] have described experiments which have been interpreted in terms of inhibition by sulfite, bisulfite and phenylsulfinate ions. In view of the fact that in the above experiments phosphate ion was also present as was sodium and potassium ion it would appear that the conclusions of these author's require modification. This also appears to be true of the recent studies of Laidler and Hoare[35,36,54] on the molecular kinetics of the urease-urea system, where the possibility of interaction of the buffer components with the enzyme or enzyme-substrate complex was not considered, and where the exact nature of the buffer used was not specified.

Although Harmon and Niemann[33] obtained straight line plots in their study of phosphate as a competitive inhibitor, examination of points for the 0.38 M phosphate line leads

one to suggest that a parabolic curve would be more suitable. This would indicate inhibition by sodium at high concentrations, although a mixture of dipotassium hydrogen phosphate and potassium dihydrogen phosphate was stated as the buffer constituents. On re-examination of the data[1] for this work it has been found that whereas dipotassium hydrogen phosphate and potassium dihydrogen phosphate were used in the greater part of this study, in the phosphate inhibition experiments the buffer used was actually a mixture of disodium hydrogen phosphate and potassium dihydrogen phosphate. The significance of the difference between the two mixtures was not appreciated at that time. Therefore the curvature of this line is in accordance with the work reported herein.

Kinetic and Theoretical Interpretation of Results

The results for 25°, pH 7.0, and potassium phosphate buffer obey Michaelis-Menten kinetics in so far as straight line plots of $\frac{1}{v}$ versus $\frac{1}{(S)}$ are obtained, but for sodium phosphate buffer at high concentrations considerable deviations are indicated, the curves being parabolic.

Three of the mechanisms considered in Section 1, Types B, C, and D, would satisfy the requirement of a curved plot of $\frac{1}{v}$ versus $\frac{1}{(S)}$. In B the active complex is ESA. With the condition that $A = \alpha S$ equation (25) was derived thereby yielding a parabolic function. Type C, ESS active complex, immediately yields a curve as does Type D, ESSA

1. K. M. Harmon's Laboratory notebook, page 53, May 14, 1948, kindly loaned by Dr. C. Niemann.

active complex, at constant activator concentration. Thus it would be impossible to distinguish between the above three mechanisms if the imposed conditions existed in vitro.

However it does not seem plausible that an enzyme would show a change in modus operandi due to different inhibitors. Therefore the following is offered in an attempt to explain how these different mechanisms could appear to interchange (i.e., Michaelis-Menten kinetics and any of the above three types).

Consider the active species as ESS. Then,

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad (1)$$

$$ES + S \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ESS \qquad (2)$$

If the rate determining step is (2), the $\frac{1}{v}$ versus $\frac{1}{(S)}$ plot would produce a parabolic curve. However if the rate determining step is (1), the plot would produce a straight line[1]. Thus the possibility exists that the rate determining step is (1) in the presence of potassium ion and (2) in the presence of sodium ion.

The apparent inhibition by sodium and potassium ions could possibly be due to either of two species, the unhydrated ion or the hydrated ion. If the attack were specific, e.g.,

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1. The author is indebted to Dr. V. Schomaker, California Institute of Technology, Pasadena, California, for fruitful discussions on this subject.

attacking the same site as the substrate, the smaller sodium ion[87] could be expected to be more effective than the larger potassium ion. However if the inhibitor is held on the enzyme surface by electrostatic charges, thus preventing complex formation by steric hindrance, the larger hydrated sodium ion[86] would be expected to be more effective, thus offering an explanation for the observed greater inhibition of sodium ion.

The simultaneous activation and inhibition of the urease-urea system by sodium or potassium phosphate may be represented by an arbitrary plot such as that given in Figure VII. It is seen that the sum of the two opposing effects is gradually altering the activity of the enzyme. At first on simultaneous increases of the concentration of both activator and inhibitor, the accelerator causes the most pronounced effect, i.e.,increased activity, but on further concentration increases of both species the inhibitor outweighs the effect of the activator and the net effect is greater inhibition. As the sodium ion is the most effective inhibitor, its inhibition plot has a larger curvature than the potassium inhibitor plot. Consequently the shift from overall activation to overall inhibition takes place at a lower sodium buffer concentration than potassium buffer concentration. This is an experimentally observable phenomenon.

The position of the minimum in the curve representing the greatest activity, (the net effect of activation and inhibition), appears to be dependent upon the concentration of

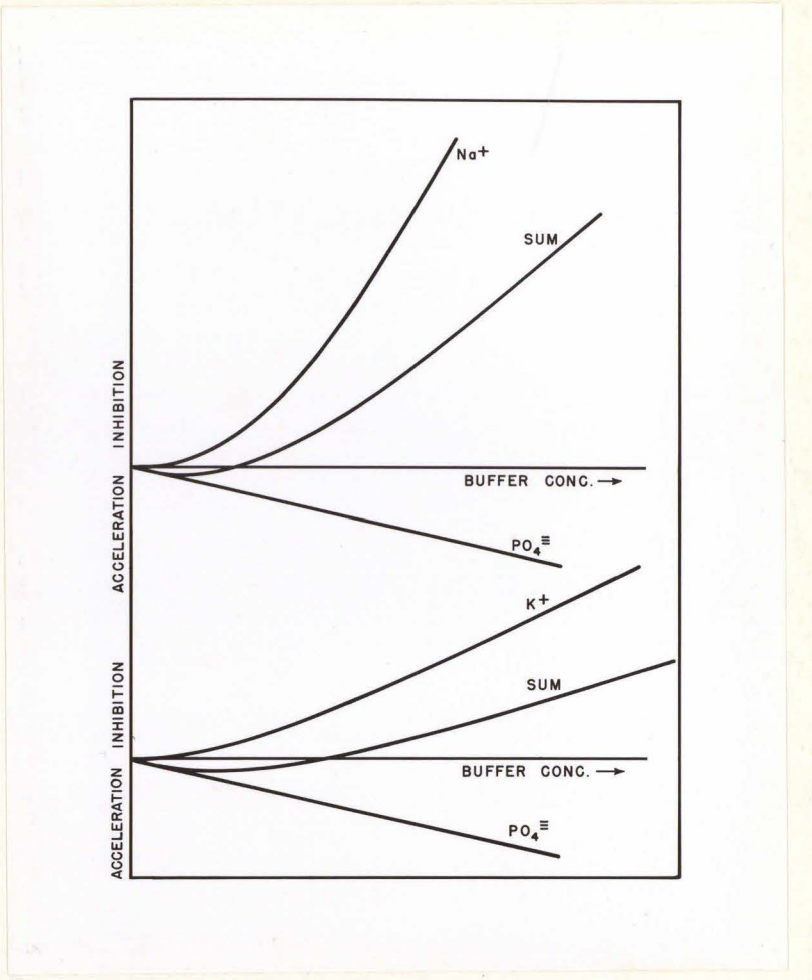


Fig VII. Schematic representation of activation and inhibition of urease by sodium and potassium phosphate.

the enzyme, and if the concentration of the enzyme is such as to permit observations to be made only on the ascending branch of the summation curve, it is possible, with potassium phosphate buffers where the degree of curvature of this curve appears to be slight, that one could be led to the erroneous conclusion that phosphate is a competitive inhibitor of this enzyme[33]. From information now at hand it is obvious that the term "specific activity" or any of its synonyms, has no general meaning when applied to urease and that a valid analysis of the kinetics of the urease-catalyzed hydrolysis of urea not only requires recognition of the phenomenon of activation by certain anions and inhibition by certain cations, but also knowledge of the concentration of all the reacting species. Thus it cannot be concluded that the value of 0.16 M potassium phosphate, at which maximum activity of urease was observed in the set of experiments reported in this thesis (cf. Figure V) has any independent significance.

It was earlier noted by Peterson, Harmon and Niemann[34] that the "specific activity" of urease, when determined in systems containing phosphate, increased upon dilution and this observation has since been confirmed in two different laboratories[38,67]. It was originally suggested[34] that the so-called dilution effect was a consequence of either dissociation of urease with an increase in the number of reactive sites, or the dissociation of a urease-inhibitor complex with the inhibitor assumed to be of natural origin or that added during crystallization. With the recognition that certain ions can apparently function as inhibitors and

others as activators of urease, it appears that the dilution effect might possibly be the consequence of a change in the relative concentrations of enzyme, indigenous activator and indigenous inhibitor[89,90].

Although these suggestions have been put forth in an attempt to correlate the observed phenomena reported herein, it is to be stressed that the urease-urea system is evidently highly complicated and that any theory dependent upon a limited knowledge of it probably will prove to be insufficient.

c. Experimental and Data

Reagents

The stock 0.1, 0.2, 0.3, 0.4 and 0.5 M buffer solutions were prepared from reagent grade dipotassium hydrogen phosphate and potassium dihydrogen phosphate and from the corresponding sodium salts. In every case irrespective of the concentration of the buffer, the pH of the solution after final dilution was 7.0 ± 0.02 at 25°. A 1.0 M stock solution was prepared daily from urea which had been recrystallized from ethanol. The crystalline urease was prepared from Arlington jackbean meal by the method of Dounce[91], all operations subsequent to the initial extraction being conducted at 5°. The thrice recrystallized urease obtained from 400 gm. of meal was dissolved in 5 ml. of water 1% saturated with hydrogen sulfide at 0° and this stock solution stored at 5°. The water used for the dilution of the enzyme stock solution was also 1% saturated with hydrogen sulfide

at 0°. The water used for all solutions was redistilled from an all-glass apparatus.

Procedure

In general the procedure used was a modification of that described by Van Slyke and Cullen[27] in which the aeration step was eliminated and the ammonia determined by the method of Conway[92]. In practice 2.0 ml. aliquots of one of the above buffer solutions were placed in eight 5.0-ml. volumetric flasks, 1.0 ml. of 0.016, 0.020, 0.028, 0.032, 0.040, 0.060, 0.10 and 0.20 M urea solution added to successive flasks and the latter placed in a bath at 25 ± 0.02°. After thermal equilibrium was obtained 0.78 ml. of a diluted enzyme solution was added[1] to each of the above solutions and the mixtures vigorously stirred with a rod kept in each flask. After 3 minutes 0.5 ml. of 2.0 N sulfuric acid was added[1] to each flask, the solution again stirred, the flasks withdrawn from the bath, the stirring rods washed and the volume of solution in each flask made up to 5.0 ml. The diluted enzyme solutions were prepared so as to contain approximately 1 microgram of protein nitrogen per ml. of solution. These solutions, which were 0.01 M in the appropriate buffer, were allowed to stand for 5 hours at 25° prior to use. For the determination of liberated ammonia a 1.0-ml aliquot of approximately 0.005 N hydrochloric acid containing Tashiro indicator[92] was placed in the central chamber of a Conway dish, a 1.0-ml. aliquot of one of

1. Rapidly by means of a syringe microburette[93].

the above 5.0 ml solutions placed in the outer chamber, the lid, lubricated with glycerol containing sodium hydroxide, placed in position so as to permit the rapid introduction of 1.0 ml. of saturated potassium carbonate into the outer compartment, the dish sealed, the contents in the outer compartment mixed, and the dish allowed to stand overnight at room temperature. The excess acid remaining in the central compartment was then titrated with approximately 0.005 N aqueous barium hydroxide. Suitable blanks were provided for each experiment and it was estimated that for a given set of experiments wherein the same enzyme solution was used a precision of $\pm$ 1.5% was obtained. A least squares treatment was used for the $\frac{1}{v}$ versus $\frac{1}{(S)_o}$ plots and in every case $(S)_o$ was taken as the mean substrate concentration prevailing over the 3 minute reaction time. It should be noted that the slopes of the plots given in Figures I, II, III, IV, and VI are dependent upon the concentration of active enzyme and because of the irreversible inactivation of urease with time comparisons of the slopes in the above-mentioned figures should be limited to those experiments which were preformed simultaneously, i.e., those given in any separate plot. The curve given in Figure V, which is based upon data obtained in separate experiments, was constructed by arbitrarily selecting the curve, given in Figure IV (upper plot), which has a slope of 4.7 for a phosphate concentration of 0.159 M as a standard and adjusting the coordinates of the other plots so that the slope of the curve representing 0.159 M phosphate in each of these plots was equal to 4.7.

DATA

Series 1

Tables 1 - 10

Tables 1 - 2

The Relative Effects of Sodium Phosphate and Potassium Phosphate on the Kinetics of the Urease-Catalyzed Hydrolysis of Urea at pH 7.0 and 25°.

Table 1[1]

Experiment IV-5

(a) 0.055 M PO_4-Na; (b) 0.055 M PO_4-K

Urea Conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.	
	(a)	(b)
52.90	0.389	0.252
26.45	0.567	0.490
15.88	1.24	0.702
10.58	1.36	1.08
8.47	2.78	(0.524)
7.41	2.88	1.27
6.35		1.64
5.29	3.21	2.08

Table 2[2]

Experiment IV-10

(a) 0.158 M PO_4-K; (b) 0.214 M PO_4-K; (c) 0.214 M PO_4-Na

Urea conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.		
	(a)	(b)	(c)
52.90			
26.45		0.225	0.323
15.88	0.287	0.331	0.454
10.58	0.378	0.470	0.582
8.47	0.424	0.521	0.749
7.41	0.520	0.640	0.911
6.35	0.556	0.697	0.951
5.29	0.670	0.810	1.08

1. See Figure I - upper plot.
2. See Figure I - lower plot.

Tables 3 and 4

The Relative Effects of Sodium Phosphate and Potassium Phosphate on the Kinetics of the Urease-Catalyzed Hydrolysis of Urea at pH 7.0 and 25°.

Table 3[1]

Experiment IV-11

(a) 0.214 M PO_4-K; (b) 0.267 M PO_4-K; (c) 0.267 M PO_4-Na

Urea conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.		
	(a)	(b)	(c)
52.90	0.268	0.281	0.399
26.45	0.390	0.453	0.313
15.88	0.597	0.674	0.963
10.58	0.740	0.854	1.13
8.47	0.883	1.06	1.22
7.41	1.18	1.20	1.04
6.35	1.21	1.40	1.82
5.29	1.46	1.48	2.14

Table 4[2]

Experiment IV-7

(a) 0.158 M PO_4-Na; (b) 0.158 M PO_4-K; (c) 0.108 M PO_4-K

Urea conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.		
	(a)	(b)	(c)
52.90	0.361	0.204	0.231
26.45	0.443	0.265	0.353
15.88	0.735	0.409	0.501
10.58	0.866	0.575	0.714
8.47	0.924	0.654	0.802
7.41	1.21	0.790	0.976
6.35	1.24	0.922	1.12
5.29	1.49	1.03	

1. See Figure II - upper plot.
2. See Figure II - lower plot.

Tables 5 and 6

The Relative Effects of Sodium Phosphate and Potassium Phosphate on the Kinetics of the Urease-Catalyzed Hydrolysis of Urea at pH 7.0 and 25°.

Table 5

Experiment IV-6

(a) 0.108 M PO_4-Na; (b) 0.108 M PO_4-K; (c) 0.055 M PO_4-K

Urea Conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.		
	(a)	(b)	(c)
52.90	0.255	0.244	0.424
26.45	0.358	0.349	0.649
15.88	0.522	0.439	1.02
10.58	0.710	0.595	1.72
8.47	0.995	0.826	2.09
7.41	1.04	0.891	2.16
6.35	1.37	1.11	2.24
5.29	1.46	1.31	3.35

Table 6

Experiment IV-1

(a) 0.267 M PO_4-Na; (b) 0.267 M PO_4-K; (c) 0.055 M Maleate-K

Urea Conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.		
	(a)	(b)	(c)
52.90	4.12	2.91	2.53
26.45	7.88	4.32	3.27
15.88	12.88	8.43	3.86
10.58	18.13	12.40	
8.47	25.82	9.79	4.97
7.41	30.24	20.10	4.53
6.35	43.75	21.37	6.74
5.29		21.37	8.23

Table 7

The Effect of Potassium Chloride on the Urease-Urea System in a Potassium Phosphate Buffer at pH 7.0 and 25°[1].

Experiment IV-14

(a) 0.055M PO_4-K; (b) 0.055M PO_4-K, 0.10M KCl

Urea conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.	
	(a)	(b)
15.88	0.253	0.381
10.58	0.350	0.381
8.47	0.372	0.513
7.41	0.566	0.635
6.35	0.660	0.750
5.29	0.784	0.900

Table 8

The Effect of Sodium Chloride on the Urease-Urea System in a Sodium Phosphate Buffer at pH 7.0 and 25°[2].

Experiment IV-15

(a) 0.055 M PO_4-Na; (b) 0.055 M PO_4-Na, 0.10 M NaCl; (c) 0.055 M PO_4-Na, 0.265 M NaCl

Urea conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.		
	(a)	(b)	(c)
52.90	0.332	0.420	0.606
26.45	0.733	0.856	1.16
15.88	1.34	1.724	2.03
10.58	1.51	2.73	2.93
8.47	1.77	2.51	3.03
7.41	2.53	5.91	3.57
6.35	3.53	4.93	4.02
5.29	3.95	5.10	5.52

1. See Figure III - lower plot.
2. See Figure III - upper plot.

Tables 9 and 10

The Effect of Varying the Concentration of Potassium Phosphate Buffer on the Kinetics of the Urease-Urea system at pH 7.0 and 25°.

Table 9[1]

Experiment IV-9

(a) 0.055 M PO_4-K; (b) 0.108 M PO_4-K; (c) 0.158 M PO_4-K

Urea Conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.		
	(a)	(b)	(c)
52.90	0.265	0.207	0.213
26.45	0.452	0.285	0.302
15.88	1.20	0.502	0.439
10.58	1.33	0.696	0.645
8.47	1.58	0.811	0.711
7.41	2.06	0.912	0.781
6.35	2.50	1.06	0.898
5.29		1.13	1.11

Table 10[2]

Experiment IV-13

(a) 0.158 M PO_4-K; (b) 0.214 M PO_4-K; (c) 0.267 M PO_4-K

Urea Conc. x 10^6 moles/ml	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.		
	(a)	(b)	(c)
26.45		0.220	0.244
15.88	0.255	0.306	0.367
10.58	0.364	0.428	0.513
8.47	0.454	0.523	0.577
7.41	0.539	0.626	0.678
6.35	0.633	0.734	0.797
5.29	0.677	0.803	0.843

1. See Figure IV - lower plot.
2. See Figure IV - upper plot.

Series 2

Tables 11 - 14

Tables 11 and 12

The Effect of Varying the Concentration of Potassium Phosphate Buffer on the Kinetics of the Urease-Urea System at pH 7.0 and 25°.

Table 11

Experiment 11-1,2,3,4

1. 0.055M; 2. 0.108M; 3. 0.158M; 4. 0.214M PO_4-K

Urea conc. x 10^6 moles/ml.	$\frac{1}{v}$ moles x 10^6 NH_3/ml./min.			
Expt.	1.	2.	3.	4.
52.90	1.13	0.865	0.617	0.817
26.45	1.92	1.20	0.972	1.16
15.88	2.67	1.89	1.09	1.75
10.58		2.67	1.86	2.19
8.47	4.18	3.42	2.04	2.72
7.41	4.18	3.26	2.33	2.56
6.35	5.75	4.05	2.52	3.37
5.29	3.89	4.52	3.44	3.39
4.23	4.80	4.70	3.51	5.96

Table 12

Experiment II-5

(a) 0.055 M PO_4-K; (b) 0.108 M PO_4-K

Urea conc. x 10^6 moles/ml.	$\frac{1}{v}$ moles x 10^6 NH_3/ml./min.	
	(a)	(b)
52.90	1.55	0.694
26.45	2.76	1.02
15.88	4.02	1.62
10.58	5.46	2.27
8.47	7.01	2.81
7.41	7.28	3.40
6.35	11.30	3.24
5.29	7.97	

Tables 13 and 14

The Effect of Varying the Concentration of Potassium Phosphate Buffer on the Kinetics of the Urease-Urea System at pH 7.0 and 25°.

Table 13

Experiments II-6,7,8

(a) 0.055 M; (b) 0.108 M; (c) 0.158 M; (d) 0.158 M (e) 0.214 M; (f) 0.267 M PO_4-K

Urea conc. x 10^6 moles/ml.	$\frac{1}{v}$ moles x 10^6 NH_3/ml./min.					
	(a)	(b)	(c)	(d)	(e)	(f)
52.90	0.696	0.482				
26.45	1.66	0.678	0.507	0.552	0.520	0.518
15.88	2.30	1.15	0.658	0.694	0.686	0.685
10.58	3.49	1.32	1.04	0.978	0.917	0.877
8.47	5.34	1.89	1.17	1.20	1.12	1.07
7.41		2.21	1.34	1.30	1.16	1.22
6.35	5.03	2.18	1.58	1.69	1.36	1.31
5.29	7.04	2.51	1.58	1.75	1.83	

Table 14

Experiment II-9

(a) 0.055 M PO_4K; (b) 0.108 M PO_4-K

Urea Conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.	
	(a)	(b)
52.90	1.07	0.587
26.45	1.54	0.810
15.88	1.82	1.04
10.58	2.64	1.60
8.47	3.90	1.91
7.41	3.82	1.88
6.35	4.97	2.74
5.29	5.32	2.79

Tables 15 and 16

Summary of the Effect of Varying the Potassium Phosphate Concentration on the Kinetics of the Urease-Catalyzed Hydrolysis of Urea at pH 7.0 and 25°. Concentration versus Slope.

Table 15

Series 1

Expt. No.	Buffer Conc.	Corrected Slopes[1]
IV-6	0.055 M PO_4-K	14.8
	0.108	5.3
IV-7	0.108	5.3
	0.158	4.9
IV-9	0.055	14.8) Taken
	0.108	5.3) as
	0.158	4.7) Standard
IV-10	0.214	5.2
	0.158	4.7
IV-11	0.267	7.5
	0.214	6.7
IV-13	0.158	4.7
	0.214	5.5
	0.267	6.5

Table 16

Series 2

Expt. No.	Buffer Conc.	Slope of Curve
II-7	0.055 M PO_4-K	28.88
II-8	0.108	12.02
II-8	0.158	7.354
II-7	0.158	8.112
II-6	0.214	7.781
II-6	0.267	6.12

1. Original Slopes taken from Table 24.

Table 17

Slope Ratios, $\frac{K_S}{V}$, for Potassium Phosphate Buffers[1].

Expt. No.	$\frac{0.055\ M}{0.108\ M}$	$\frac{0.108\ M}{0.158\ M}$	$\frac{0.158\ M}{0.214\ M}$	$\frac{0.214\ M}{0.267\ M}$
II-1,2,3,4	1.57	1.58	0.90	
II-5	2.20			
II-6,7,8	2.40	1.48	1.27	
II-9	1.72			
IV-6	2.86			
IV-7		1.09		
IV-9	2.82	1.10		
IV-10			0.91	
IV-11				0.96
IV-13			0.85	0.85

1. See Table 24 for slope values.

Tables 18 and 19

The Effect of Varying the Concentration of Potassium Maleate on the Kinetics of the Urease-Urea System at pH 7.0 and 25°.

Table 18

Experiments III-2,3

(a) 0.055 M; (b) 0.108 M; (c) 0.158 M; (d) 0.214 M Maleate-K.

Urea Conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.			
	(a)	(b)	(c)	(d)
52.90	1.24	0.927	0.860	0.950
26.45	1.27	1.04	0.883	0.992
15.88	1.59	1.17	1.07	1.10
10.58	1.45	1.17	1.14	1.25
8.47	1.69	1.35	1.25	1.23
7.41	2.01	1.44	1.36	1.30
6.35	2.12	1.52	1.41	1.40
5.29	1.91	1.58	1.55	1.43

Table 19

Experiment III-4

(a) 0.055M; (b) 0.108 M; (c) 0.158 M Maleate-K

Urea Conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.		
	(a)	(b)	(c)
52.90	0.633	0.428	0.439
26.45	0.648	0.489	0.478
15.88	0.696	0.519	0.550
10.58	0.788	0.556	0.669
8.47	0.804	0.646	0.726
7.41	0.844	0.746	0.746
6.35	0.953	0.744	0.839
5.29	1.02	0.760	0.907

Tables 20 and 21

The Effect of Varying the Concentration of Sodium Phosphate Buffer on the Kinetics of the Urease-Urea System at pH 7.0 and 25°.

Table 20[1]

Experiment IV-8

(a) 0.055 M PO_4-Na; (b) 0.158 M PO_4-Na; (c) 0.108 M PO_4-Na

Urea conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.		
	(a)	(b)	(c)
52.90	0.292	0.439	0.218
26.45	0.493	0.527	0.343
15.88	1.10	0.603	0.488
10.58	1.45	0.968	0.627
8.47	2.02	0.893	0.820
7.41	1.66	1.19	0.963
6.35	2.18	1.44	0.976
5.29	2.88		

Table 21[2]

Experiment IV-12

(a) 0.158 M PO_4-Na; (b) 0.214 M PO_4-Na; (c) 0.267 M PO_4-Na

Urea conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min.		
	(a)	(b)	(c)
52.90	0.290	0.360	0.403
26.45	0.426	0.648	0.747
15.88	0.618	0.812	1.32
10.58	0.939	0.887	1.87
8.47	1.36	1.70	2.66
7.41	1.68	2.53	3.10
6.35	2.33	3.42	5.61
5.29	2.78	4.80	10.81

1. See Figure VI - lower plot.
2. See Figure VI - upper plot.

Tables 22 and 23

The Effect of Varying the Concentration of Sodium Phosphate Buffer on the Kinetics of the Urease-Urea System at pH 7.0 and 25°.

Table 22

Experiment III-5

(a) 0.055 M PO_4-Na; (b) 0.108 M PO_4-Na; (c) 0.158 M PO_4-Na

Urea Conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min. (a)	(b)	(c)
52.90	3.47	1.76	1.85
26.45	6.44	2.59	2.71
15.88		3.81	3.41
10.58	17.61	4.18	5.22
8.47	22.20	5.14	5.64
7.41		6.12	8.05
6.35		5.36	7.55
5.29		8.64	10.09

Table 23

Experiment III-6

(a) 0.055 M PO4-Na; (b) 0.108 M PO_4-Na; (c) 0.158 M PO_4-Na

Urea Conc. x 10^6 moles/ml.	$\frac{1}{v}$ x 10^6 moles NH_3/ml./min. (a)	(b)	(c)
52.90	4.95	2.21	2.31
26.45	8.27	3.09	2.94
15.88	12.72	4.93	4.45
10.58	25.44	4.81	5.66
8.47	23.94	6.35	2.56
7.41	29.79	8.38	7.25
6.35	29.79	9.39	11.17
5.29	23.94	14.77	10.81

Table 24

Slope, Intercept K_S' and K_S Values

Experiment II, III, IV

Expt. No.	Buffer Conc.		Slope	Intercept	$K_S'(M)^1$	$K_S(M)$
II-1,2,3,4	0.055M	PO_4-K	33.42	0.425	0.08	
	0.108	"	21.95	0.469	0.05	
	0.158	"	13.89	0.378	0.04	
	0.214	"	15.38	0.644	0.02	0.10
II-5	0.055M	PO_4-K	50.02	0.183	0.27	
	0.108	"	22.42	0.188	0.12	0.004-0.003
II-7	0.055M	PO_4-K	28.88	0.511	0.06	
8	0.108	"	12.02	0.288	0.04	
8	0.158	"	7.354	0.245	0.03	
7	0.158	"	8.112	0.195	0.04	
6	0.214	"	7.781	0.161	0.05	
6	0.267	"	6.12	0.301	0.02	
II-9	0.055M	PO_4-K	28.86	0.173	0.17	
	0.108	"	16.84	0.351	0.05	
III-2	0.055M	Maleate-K	5.16	1.12		
	0.108	"	3.85	0.87		
3	0.158	"	3.89	0.77		
	0.214	"	2.74	0.91		0.004
III-4	0.055M	Maleate-K	2.09	0.568		
	0.108	"	1.79	0.409		
	0.158	"	2.19	0.426		0.003
IV-5	0.055M	PO_4-Na	18.19	0.024	0.76	
	0.055M	PO_4-K	9.53	0.076	0.13	
IV-6	0.055M	PO_4-K	16.56	0.025	0.66	
	0.108	"	5.82	0.082	0.07	
	0.108M	PO_4-Na	6.90	0.086	0.08	
IV-7	0.108M	PO_4-K	5.01	0.172	0.029	
	0.158	"	4.58	0.096	0.048	
	0.158M	PO_4-Na	6.13	0.248	0.025	
IV-8	0.108M	PO_4-Na	4.02	0.224	0.018	
	0.158	"	6.25	0.104	0.06	
	0.055	"	13.81	0.055	0.25	
IV-9	0.055M	PO_4-K	14.84	-0.029	-0.512	
	0.108	"	5.28	0.121	0.04	
	0.158	"	4.67	0.144	0.03	

Table 24 (cont)

Expt. No.	Buffer Conc.	Slope	Intercept	K_S'(M)[1]	K_S(M)
IV-10	0.214M PO_4-Na	4.68	0.144	0.03	
	0.214 M PO_4-K	3.40	0.095	0.04	
	0.158 "	3.11	0.020	0.16	
IV-11	0.267M PO_4-K	6.82	0.189	0.04	
	0.214 "	6.54	0.132	0.05	
	0.267M PO_4-Na	9.44	0.223	0.04	
IV-13	0.158M PO_4-K	3.05	0.044	0.07	
	0.214 "	3.57	0.064	0.06	
	0.267 "	4.18	0.056	0.08	
IV-14	0.055M PO_4-K	3.97	0.033	0.01	
	0.055 & 0.03M KCl	5.13	-0.172	0.03	
	0.055 & 0.10M KCl	3.93	-0.073	0.05	

1. K_S' = Apparent K_S.

Part 1

Section 3

A Study of the Reactivation of Dialyzed Urease by Salts

A Study of the Reactivation of Dialyzed Urease by Salts

a. Introduction

The regulatory mechanisms of enzyme activity have received much attention, and the controlling variables may be listed as, 1. concentration of substrate, 2. concentration of enzyme, 3. temperature, 4. pH, and 5. co-enzyme and anti-enzyme[78,56]. Doubt can be cast on the degree to which the first four of these are operative in vivo. For in considering any one specific site in vivo, very little rigorous control can be brought about in these variables. However, the last of these variables, co-enzyme and anti-enzyme, have a much larger scope in their applicability for delicate control of enzyme activity.

As the cell membrane is known to exhibit differential diffusion properties, it can easily be seen how various ions entering and leaving the cell could activate or inhibit the dormant enzyme.

This study is believed to expose a system in which control by means of co-enzyme and anti-enzyme (inhibitors) can be found due to ions alone.

The problem of a co-enzyme for urease has often been discussed and much work was done in this field up to 1932, when Sumner[47] emphatically concluded that there was no such entity. Until this time the work of Onodera[44], Kato[45], and Rockwood[94,95] favored the existence of a co-enzyme,

while earlier work of Sumner[2] and Lovgren[46] weighed against it.

In 1915, Onodera[44] reported that the activity of (unbuffered) urease solutions disappeared on five days dialysis, but could be restored, and even enhanced, by small subsequent additions of fresh urease; neither the diffusate nor a mixture of diffusate and dialysate showed urease activity. This behavior Onodera attributed to the presence in the urease preparation of an essential coenzyme, which, at least an essential part, was destroyed by dialysis.

Lovgren[46] in 1921 attempted to confirm Onodera's work using sodium phosphate buffers and concluded his evidence was not compatible with Onodera's theory. He did show however that the dialysate produced greater hydrolysis than did the diffusate, but together produced less than the sum of the individual components. He claimed that Onodera's results could be explained on the basis of a pH effect.

In 1923 Kato[45] concluded that in crude urease there were two components, enzyme proper and a further essential substance X, which with urea formed a compound that could be hydrolyzed by urease. Some of Kato's experiments were carried out in a mixture of sodium phosphate and potassium phosphate buffers and others without buffers.

Rockwood[94,95] in 1923-4 found that of forty-eight compounds tested, including amino acids and amines, many activated urease. A highly buffered medium (0.5 M potassium phosphate) having been used, the effects could not be attributed to pH changes.

Jacoby and Sugga[48] had previously reported that urease lost its activity during dialysis.

When Sumner[2] crystallized urease in 1926, he stated that he believed that no co-enzyme existed for urease, for the greater the purification by recrystallization, the greater the activity of the preparations. However, in 1930 Haldane[78] again discussed the co-enzyme possibility. This prompted Sumner[47] to investigated the problem once again. For a sodium potassium phosphate mixed buffer medium, he found a definite decrease in activity on dialysis which could not be restored to any degree by the addition of fresh urease, and he explained Ondera's and Kato's work on the basis that the inactivated urease formed during dialysis acts both as a buffer and as a protective colloid against traces of heavy metals.

The activation problem has many other aspects. Hellerman[62,85,97] discussed the role of the sulfhydryl groups with respect to metal ion inactivation, Sizer[64,65] studied their activity as a function of the oxidation-reduction potential, and Desnuelle[63] showed the existence of various types of sulfhydryl groups in the molecule, on some but not all of which the activity was dependent. Armstrong[28] and Kato[45] demonstrated activation by glycine and Hellerman[62], by cysteine, hydrogen sulfide, and thioglycollate; Sumner[71] discusses many other examples including gum arabic and egg albumin. He suggested that these and many other "auxo" substances[94,96] act as protective agents and buffering components.

Golby[40] reported that sodium dodecylsulfonate greatly depressed urease activity and trimethyl dodecyl ammonium chloride caused a very slight depression.

In Part 1, Section 2, of this thesis the effects of buffer systems on the catalysis of urea hydrolysis by urease were investigated and it was concluded that both components of the buffer affect the system in a specific manner. It was shown that the activity of urease differed in sodium and potassium phosphate buffers. To investigate further the activity with a change of anion, ethylenediamine was used as the cation. It was further hoped that an insight into which component of the buffer acted as the activator and which as the inhibitor might be obtained.

b. Résumé of Experimental Results

A. Dialyzed Urease

1. Upon dialysis the activity of urease is raised above the initial value, the order of activation for the buffers investigated is ethylenediamine phosphate > sodium-potassium phosphate > ethylenediamine hydrochloride.

2. For a series of ethylenediamine salts the order of activation is citrate > phosphate > maleate > sulphate > chloride > acetate.

3. At constant phosphate concentration, 0.3 M, with both 0.28 M ethylenediamine and 0.59 M tris (hydroxymethyl) aminomethane the activity remains constant, but at a constant ethylenediamine concentration with a variation of anions the activity varied. This observation is offered as circumstantial evidence that the phosphate ion might possibly be the

activating species in the ethylenediamine phosphate buffer.

4. With increasing concentrations of the anions chloride and phosphate, added as their ethylenediamine salts, the activity is observed to increase.

5. At equivalent concentrations of phosphate, ethylenediamine phosphate produces three times as much activation as does sodium-potassium phosphate.

6. The kinetics of the dialyzed urease catalyzed hydrolysis of urea appear to be amenable to the Michaelis-Menten treatment.

B. Undialyzed Urease

7. Undialyzed urease appears to obey Michaelis-Menten kinetics in the two buffer systems ethylenediamine hydrochloride and ethylenediamine phosphate.

8. The relative order of activation by the ethylenediamine salts was found to be the same as for dialyzed urease with the exception acetate $>$ chloride.

c. Discussion of Results

Studies on the Reactivation of Dialyzed Urease at pH 7.0 and 25°

Due to cognizance of the possibility that urease in vivo may be complexed with both an indigenous inhibitor and an activator, or that these could have been added during the crystallization procedure, dialysis experiments were carried out with the intent of removing both species, anion and cation, and then reactivation was attempted by the addition of salt containing an activator. To minimize denaturation during the dialysis, the urease was dialyzed against the same

buffer solution in which it was dissolved, 0.001 M ethylenediamine hydrochloride.

From the data in Figure VIII and Tables 25 and 26 (also see Tables 27, 28, 29, 30) it is seen that it is possible to reactivate the enzyme, after removal of the inhibitor and coenzyme by dialysis, by the addition of an activator, to a greater degree of activity than displayed before dialysis. The time required for removal of the inhibiting species and to reach maximum activity is variable, depending on the state of the initial urease solutions. Variations between one and five days were observed. A variation of time was also noted with various buffers used to restore activity. The phenomenon of a rise of activity after initial dialysis was observed with both urease dissolved in water and dialyzed versus water (cf. Table 27) and urease dissolved in a weak buffer solution and dialyzed versus a weak buffer solution (0.001 M) (cf. Tables 25, 26, 28, 29, 30). This phenomenon was observed using the buffers ethylenediamine hydrochloride, ethylenediamine phosphate and sodium-potassium phosphate, in the enzyme solutions. The order of activation for equal concentrations of buffer (e.g., anion) was found to be ethylenediamine phosphate sodium-potassium phosphate ethylenediamine hydrochloride.

Figure VIII, upper plot, demonstrates the possibility that the activator and inhibitor present in the intact enzyme do not dialyze out at the same rate. The activator appears to dialyze out first, causing a decrease in activity and then as the inhibitor dialyzes out an increase in activity

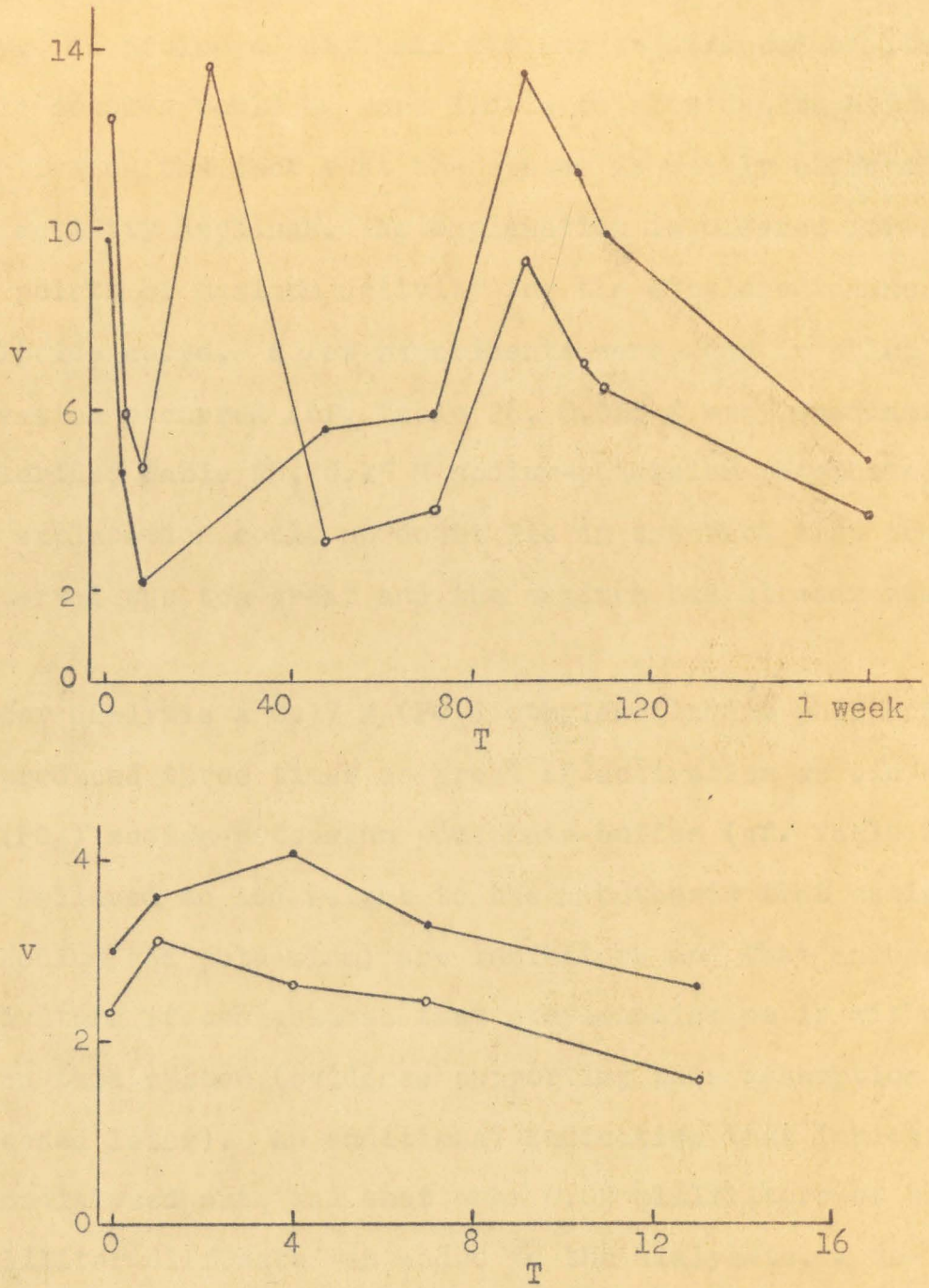


Figure VIII
Reactivation of Urease After Dialysis

v in micromoles of ammonia/ml/three minutes: Upper plot, T, time dialyzed in hours (last point one week); solid circles- 0.15M sodium-potassium phosphate, open circles -0.015 M ethylenediamine hydrochloride. Lower plot, T, time dialyzed in days, solid circles- 0.3 M ethylenediamine phosphate, open circles- 0.015 M ethylenediamine hydrochloride.

is observed which continues to a point beyond the initial activity. On prolonged dialysis the enzyme stripped of its inorganic complex would be more liable to slow denaturation, irrespective of the fact that the system is weakly buffered, and the activity declines. No explanation is offered for the two points of maximum activity for the ethylenediamine hydrochloride curve. A few experiments were made in which no activation occurred (cf. Table 26, 0.015 M ethylenediamine hydrochloride; Table 29, 0.15 M sodium-potassium phosphate) and the explanation could no doubt lie in the fact that the time interval was too great and the maximum had already been passed.

After dialysis a 0.17 M (PO_4) ethylenediamine phosphate buffer produced three times as great an activation as did a 0.15 M (PO_4) sodium-potassium phosphate buffer (cf. Table 29). This is believed to add weight to the hypothesis that cations (e.g., sodium and potassium) are inhibitors and that anions are activators if one assumes that ethylenediamine is without effect on this system (evidence supporting this assumption is discussed later). An additional indication that inhibitor had been dialyzed out, was that when 0.02 milliliters of the two milliliter diffusate was added to the dialysate, a decrease from 3.1×10^{-6} moles NH_3/ml./three minutes to 2.5×10^{-6} moles NH_3/ml./three minutes was observed.

Studies on Urease in the Buffer Systems, Ethylenediamine hydrochloride and Ethylenediamine phosphate at pH 7.0 and 25°.

The type of kinetics found in any particular urease-urea

system has been shown to be highly dependent on the buffer employed[32,33]. To insure that these organic-inorganic buffers were not causing a new kinetic behavior, ethylene-diamine hydrochloride series were run using dialyzed and undialyzed enzyme. Similar plots were obtained, shown in Fig. IX (Tables 31,32). The straight lines indicate that Michaelis-Menten kinetics may be valid for these systems. As the buffer concentration is increased the slope remains constant with a decrease in the intercept (Fig. IX, upper plot). If co-enzyme kinetics can be likened to the counter part of inhibition, the type of activation according to Ebersole[80] terminology would appear to be an uncompetitive type.

Ethylenediamine phosphate buffer also produced a straight line for dialyzed urease, when $\frac{1}{v}$ versus $\frac{1}{(S)}$ was plotted (cf. Table 33).

If the apparent activation by unspecific anions is reversible, it would be expected that the mass action law would hold, and with increasing concentration of anion greater activity should be observed. This much is observed (Figures X (Tables 34, 35), XI (Tables 36, 37, 38)), even though the shapes of the curves, which refer to four different dialyzed preparations, are different. Velick[88] working with aldolase, cites a situation which may be comparable. He states that

"at the isoelectric point there existed in the water of the protein solution an excess of six phosphate ions per mole of protein and a deficit of six to seven potassium ions.... If phosphate were bound by a protein in solution one would anticipate from the laws of mass action that the number of ions bound per mole would be a function of the phosphate ionic concentration."

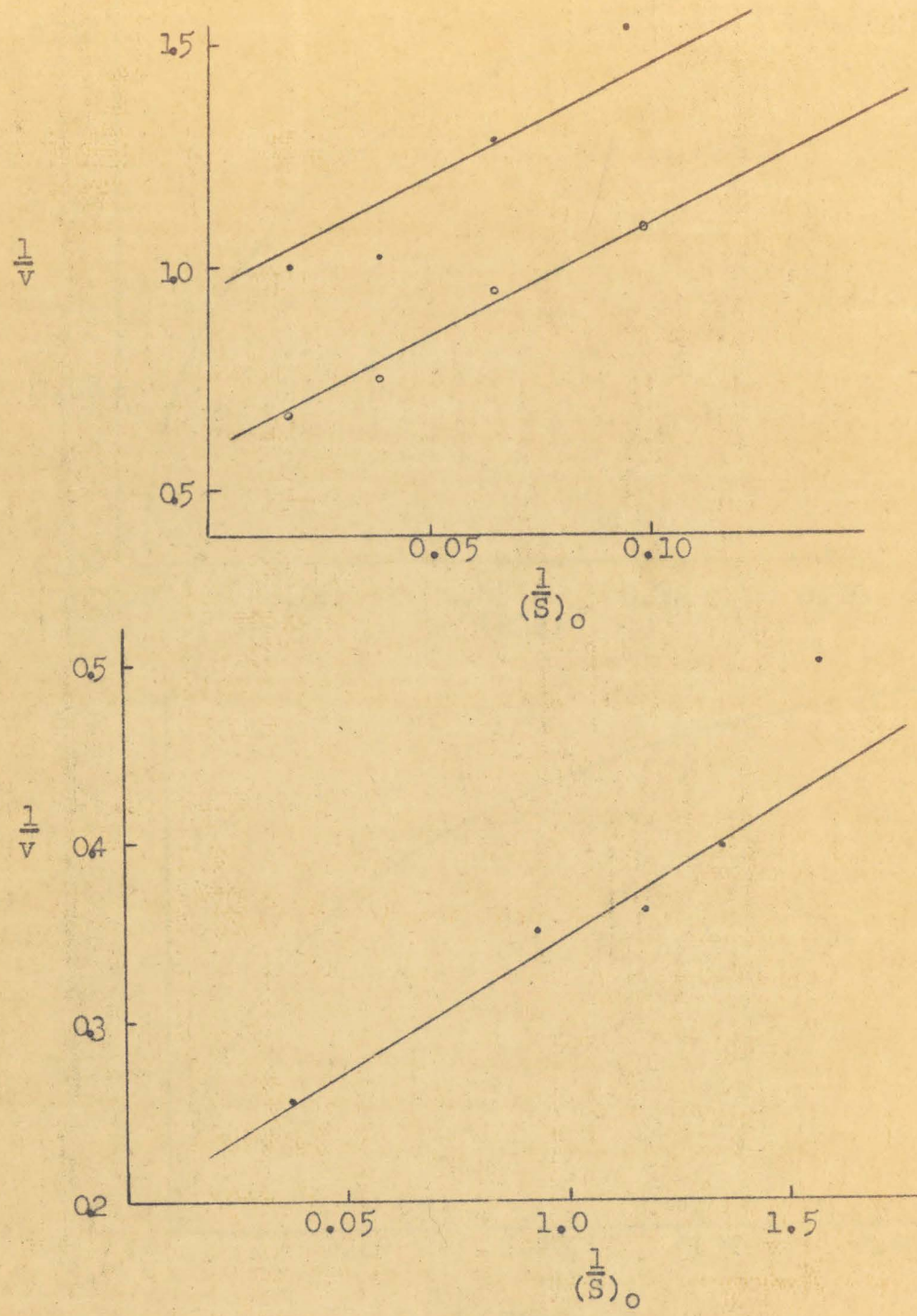
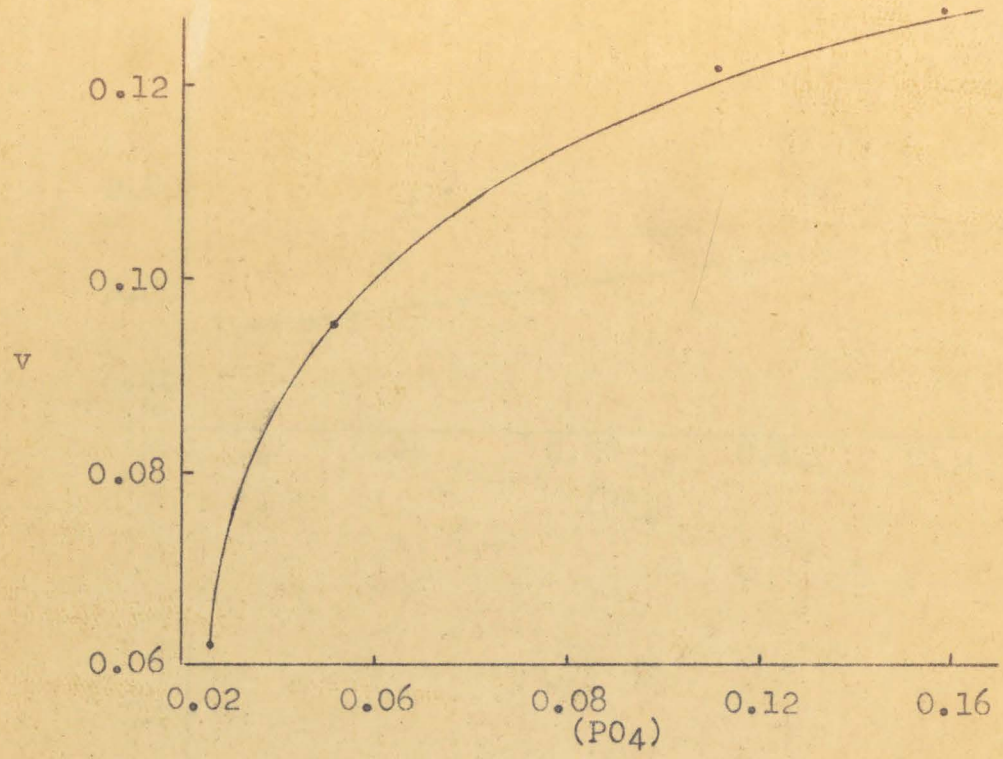


Figure IX

The Activity of Undialyzed and Dialyzed Urease in Ethylenediamine hydrochloride Buffers. $1/v$ in moles x 10^6 of ammonia/ml/minute; $1/(S)_o$ in moles x 10^6 of urea/ml. Upper plot, dialyzed, open circles-0.4 M ethylenediamine hydrochloride, solid circles-0.16 M ethylenediamine hydrochloride. Lower plot, undialyzed, ethylenediamine hydrochloride, 0.16 M.



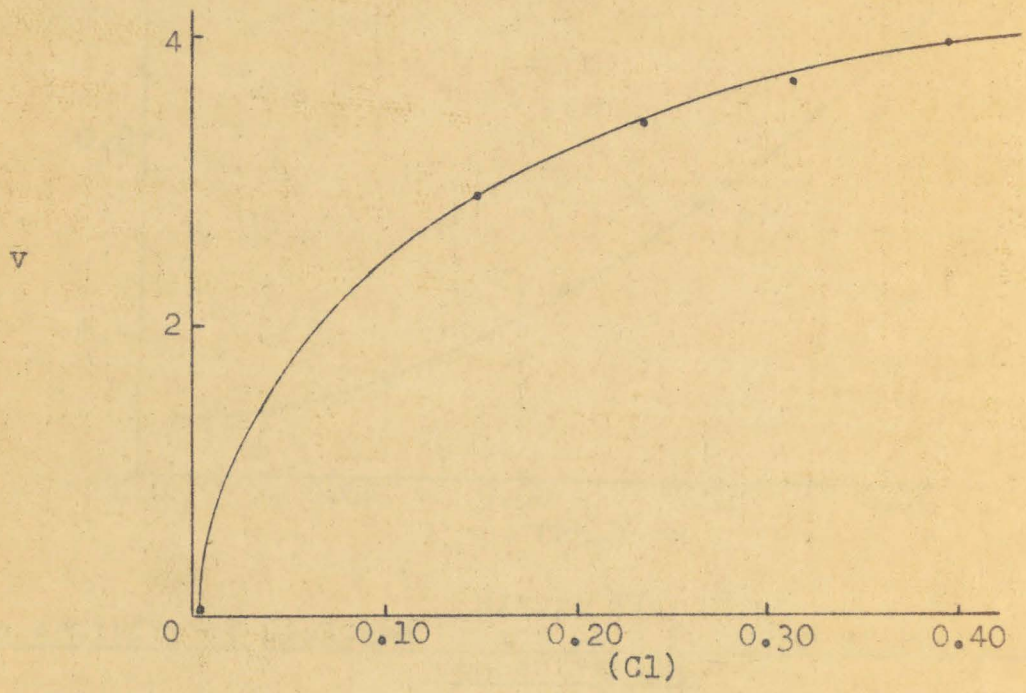


Figure X

The Activity of Dialyzed Urease as a Function of Phosphate and Hydrochloride Concentration.

v in micromoles of ammonia per ml per three minutes. Upper curve, molar ethylenediamine phosphate concentration. Lower curve, molar ethylenediamine hydrochloride concentration. Upper curve, 15 days dialysis. Lower curve, 7 days dialysis.

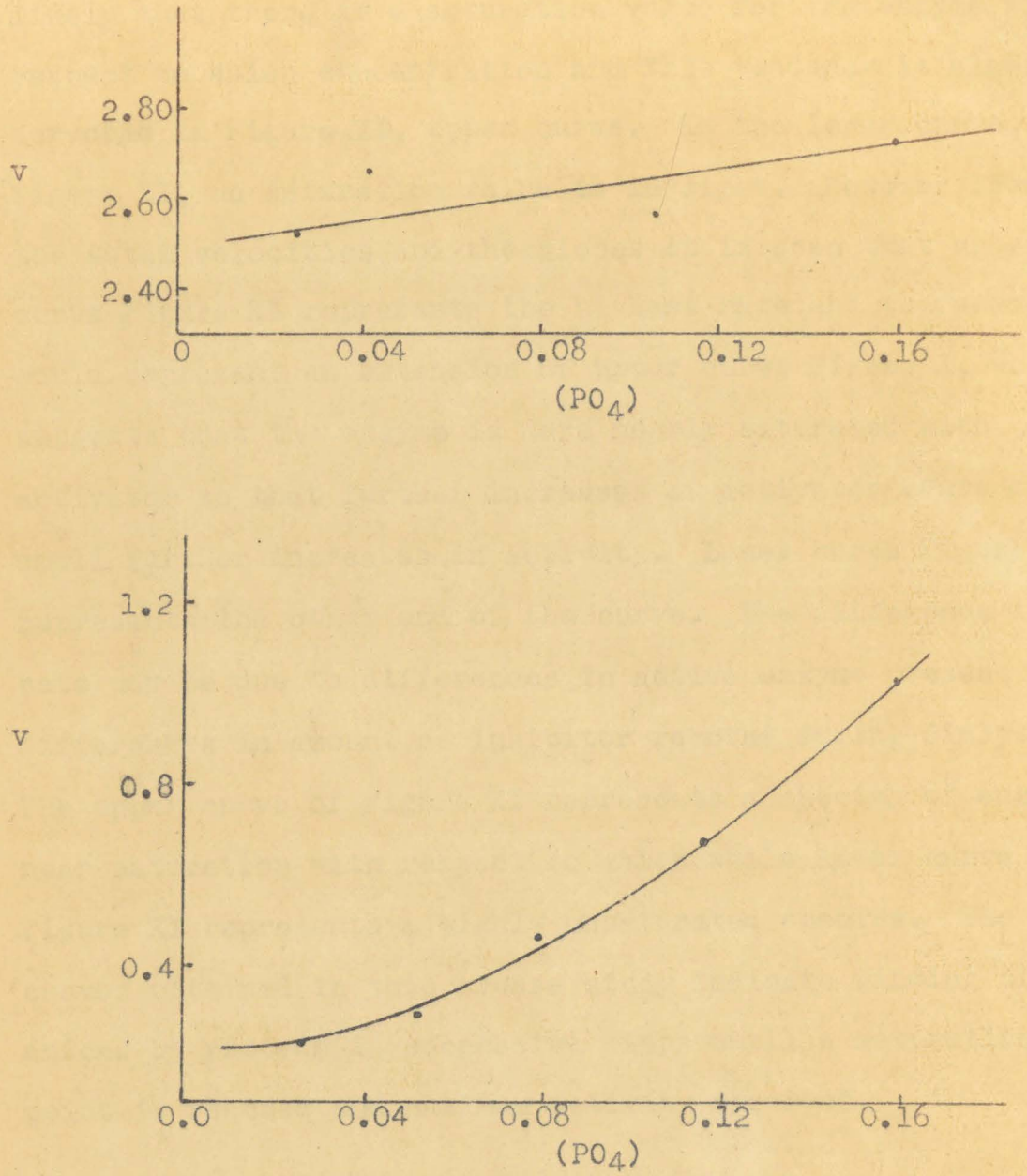


Figure XI
The Activity of Dialyzed Urease as a Function of Phosphate Concentration

v in micromoles of ammonia per ml. per three minutes; (PO_4) in moles of ethylenediamine phosphate. Upper plot, urease dialyzed thirteen days. Lower plot, urease dialyzed nine days.

This difference in curvature might possibly be ascribed to the following. From the curves in Figure X it appears likely that there is a saturation value for the enzyme with respect to anion concentration and this tendency is also observable in Figure XI, upper curve. In the lower curve, Figure XI, no saturation value is in sight. However from the total velocities and the slopes it is seen that upper curve Figure XI represents the highest rate and therefore could represent an extension of upper curve Figure X, which suggests that the enzyme is more nearly saturated with activator so that further increases of activator cause only small further increases in activity. Lower curve Figure XI represents the other end of the curve. The difference in rate may be due to differences in active enzyme present or differences in amount of inhibitor removed during dialysis. The upper curve of Figure XI represents a species of enzyme near saturation with respect to anion while lower curve Figure XI represents a highly unsaturated species. The curves obtained in this urease study indicate binding of anions by protein in successive steps until a saturation point is reached and maximum activity observed.

Studies on Dialyzed Urease in Various Buffer Systems, Varying both Anionic and Cationic Constituents at pH 7.0 and 25°.

The adopted convention of activation of urease by anionic species appears to be relatively non-specific. The relative order of activation of dialyzed urease for equal concentrations of anions as their ethylenediamine salts was found to be citrate > phosphate > maleate > sulphate > chloride > acetate (cf. Table 39). The undialyzed urease produced a similar order, Table 40, with one exception, acetate > chloride. The differences between consecutive members of the above series are greater for dialyzed urease preparations, indicating that without the removal of the inhibitor activation is not so pronounced. The observed order of activation for citrate, phosphate and acetate at pH 7.0, agrees with the results obtained by Sumner[19].

The assumption that the phosphate ion was the active constituent of the ethylenediamine phosphate buffer was based on the following observation. By keeping the phosphate concentration constant, 0.3 M, and varying the organic component the relative effect of this ion was observed. Ethylenediamine, 0.28 M, and tris(hydroxymethyl) aminomethane, 0.59 M, were used. Table 41 shows that this produced similar degrees of hydrolysis. However, by holding the concentration of the ethylenediamine constant, 0.4 M, and varying the anionic species, acetate and citrate being used, large differences of activity were observed. Series were run using both dialyzed and undialyzed urease and no difference in behavior could be

detected. An ionic strength variation can perhaps account for a slight deviation in the phosphate test, but it is unlikely the second observation can be explained on this basis. The acetate, 0.67 M, produced the expected result, greater activation, than the citrate, 0.27 M, as the concentration was much larger than the citrate and as in the phosphate case, increasing the concentration of the anion produces greater activity.

An insight into the effect of diamines on urease can be gathered from the work of Elson[43]. This work demonstrated that easily oxidized aromatic diamines cause inhibition of urease, while difficultly oxidized aromatic diamines do not. The oxidation of these diamines was assumed to take place by the simultaneous reduction of the S-S groups in the enzyme molecule. No instance of activation by the diamine was reported. Though direct comparison of aromatic and aliphatic diamines cannot be made, yet the behavior would be expected to be of the same kind but perhaps of a different degree.

It is to be pointed out again that this so-called activation by anions could possibly be a process by which the indigenous inhibitor is removed; however, the dialysis could be expected to remove this inhibitor if it were of small molecular weight.

Nature of Enzyme-Activator Complex

An insight into the nature of the enzyme-activator complex can be gathered from the work on the reactivation of

dialyzed urease. Various attacks were made to effect the greatest recombination of enzyme and co-enzyme. It was observed that (1) hydrogen sulfide interferes with the recombination, but cannot be entirely left out, and (2) low temperature retards the recombination. The hydrogen sulfide was necessary to stabilize the enzyme during the long activation period. A compromise between these factors was evolved (see experimental). The best results were obtained when the same buffer was used in the enzyme solution and reaction mixture. The proposition may be brought forth that perhaps a complex forms involving urease-co-enzyme-urea and this is formed very rapidly in the reaction mixture when an excess of co-enzyme is present. This is based on the observation that after an activation period of five hours in 0.3 M buffer, a difference in activity is observed by increasing the concentration of the buffer from 0.09 to 0.27 M in the reaction mixture. Thus the enzyme does not necessarily become saturated with co-enzyme during the activation period.

d. Discussion of Earlier Work on a Co-enzyme for Urease

In the light of the results of the work of this thesis, a re-interpretation of earlier work shows that none of these, in the manner of their execution disagrees with the inhibitor-activator theory proposed herein.

The co-enzyme postulate by Onodera[44] was the result of dialysis experiments. Upon dialyzing urease for five days the activity was significantly diminished and on the addition

of a small amount of fresh enzyme the activity could be restored to a value beyond that initially observed. He conjectured that the co-enzyme had been dialyzed free and was restored on the addition of fresh enzyme. The activation was no doubt due to the fact that the dialyzed denatured protein acted in the capacity of a protective agent (as suggested by Sumner[47]), while the dilution effect[34] on the small amount of fresh enzyme added accounted for the increase of activity. No buffer was used. The co-enzyme he postulated as being present in the fresh enzyme was claimed not to be inorganic. However when an addition of an incinerated 1% solution of urease was made to the dialyzed urease a small increased was observed, 0.13 N/10 NH_3 in 16.5 hours to 0.22 N/10 NH_3. This rise could be due to a small amount of inorganic material associated with the enzyme before incineration.

Although Lovgren[46] concluded that his attempted repeat of Onodera's work did not verify Onodera's conclusion, it is in entire agreement with the work of this thesis. From Table XVI[46] the following figures are reproduced:

1 week dialysis

(1) Dialysate	7.6 mg. NH_3	$(1)^{80°}$	0.1 mg. NH_3
(2) Diffusate	6.8 " "	$(2)^{80°}$	0.1 " "
(1) + (2)	22.2 " "		
(1) + $(2)^{80°}$	17.2 " "		
$(1)^{80°}$ + (2)	7.3 " "		

Note. $80°$ indicates heating to $80°$ before use. Buffer used M/2 sodium phosphate.

From these data he correctly concluded that the enzyme is thermolabile, not the co-enzyme as Ondera had reported. In reinterpreting these results it must first be stated that in the buffer system M/2 sodium phosphate one would not expect much buffer activation because of the high concentration of sodium ion. Thus the fact that the diffusate contained the co-enzyme anion is seen from the figures on heating. The diffusate could not be further activated on the addition of heated dialysate, but the reverse did take place. Thus the diffusable co-enzyme was to be found in the diffusate. Whether the co-enzyme which had diffused out, or the phosphate of the buffer was responsible for the increase is doubtful, but the latter seems more probable.

Kato's theory[45] of the X substance which combined with urea was evolved from his work on precipitating urease with calcium phosphate. In this manner a filtrate and a precipitate were obtained. He found that glycerol greatly increased the activity of the precipitate while only slightly affecting the filtrate.

From Table XXXVI[45]

	Without glycerol	with glycerol
Filtrate	50.8 mg. NH_3	52.8 mg. NH_3
Precipitate	56.0 " "	126.0 " "
Together	76.5 " "	94.0 " "

The precipitate no doubt contained a high concentration of calcium phosphate and the phosphate acted as the activator

while the glycerol merely served as a protecting agent[71].

Sumner[2] first rejected the idea of a co-enzyme on the basis that further purification caused greater activity. However, losses of indigenous inhibitor due to recrystallization may again be responsible. He states[70,83] that an analysis of recrystallized urease always left an ash of 1-2%. The possibility that this inorganic ash may be inhibitor-activator cannot be overlooked. Sumner's[47] latter work was done in 9.6% phosphate sodium-potassium buffers and he reported finding no difference between fresh urease and dialyzed urease. The time interval involved in the dialysis experiments could well have been such that the point of maximum activity had been passed and an addition of the buffer, containing both activator and inhibitor, did not change the activity from the previous value. Sumner[71] discusses the role of "auxo" substances and places these influences on their ability to act in the capacity of protective agents and buffering components.

The work of Golby[40] on surface active agents may fall within the postulated inhibition by sodium ion. For of the two surface active agents, the one containing sodium, sodium dodecylsulfonate caused depression of hydrolysis, while the agent trimethyldodecylammonium chloride, was found to be ineffective. This hypothesis would agree with the work of Urban and Moreland[55] who reported no effect of surface active agents on the activity of urease.

e. Miscellaneous Observations

1. It was observed that after five and a half months the urease dissolved in the 0.001 M ethylenediamine hydrochloride buffer was still active. Therefore it is concluded that this is an excellent buffer for storing urease preparations.

2. Preheating the enzyme solution to 40° before the addition of hydrogen sulfide does not aid in the recombination of urease and co-enzyme and greatly denatures the enzyme.

3. Denaturation during dialysis involves more than a pH change, for enzyme solutions kept at a pH of 7.0 during dialysis became inactive much faster than undialyzed preparations.

4. Urease does not become inactive in lypholizing.

5. Neither biuret nor thiourea is hydrolyzed by urease.

6. Further examples of inhibition by sodium chloride and potassium chloride were observed (cf. Table 29, Expt. VII-11a).

7. Preliminary experiments on undialyzed urease in ethylenediamine phosphate buffers indicated that the kinetic behavior is different than with dialyzed preparations.

8. An illustration of the dilution effect was observed.

Expt. VII - 21.

Amount of enzyme used for dilution to 100 ml.	NH_3/ml./min.
0.02 ml.	0.429
0.01 ml.	1.611

f. Experimental and Data

Reagents: The buffer solutions were prepared from C.P. reagents. Acids used were phosphoric, sulfuric, citric, acetic, hydrochloric, and maleic. The ethylenediamine was twice distilled from KOH, in a closed system attached to an aspirator, in all glass apparatus, b.p. 115°. The tris(hydroxymethyl)aminomethane was recrystallized twice from ethyl alcohol, and the maleic acid recrystallized from water. In all cases the buffers made with ethylenediamine, e.g. 0.3 M ethylenediamine hydrochloride (0.3 M ED. HCl) the molarity refers to the concentration of the anion. Stock solutions were prepared varying from 0.05 M to 0.5 M which were used in both enzyme solutions and reaction mixtures. All buffers were prepared so that on final dilution the pH = 7.00 ± 0.02 at 25°. The sodium and potassium phosphate have been described previously[1]. Crystalline urease was prepared as in Part I, Section 2; however, the final product was dissolved in 0.001 M ED.HCl and kept at 5°. The dialyzing medium was also 0.001 M ED.HCl. All solutions were made from redistilled H_2O using all glass apparatus.

Procedures: (a) Dialysis

Two types of dialysis were carried out.

I. One ml. of stock urease solution was placed in the cellulose[2] bag (see Figure XII) and 2 ml. of 0.001 M ED.HCl were placed in the outer chamber.

- -

1. Part I, Section 2 - this thesis.
2. Visking Seamless Cellulose Tubing. 1/2 inch inflated. The Visking Corporation, 6733 W - 65th Street, Chicago, 38 Illinois.

II. One ml of stock urease solution was placed in the cellulose bag, the outer chamber being discarded. The inner chamber slowly rotated in 450 ml of 0.001 M ED.HCl. The dialyzing medium was changed daily.

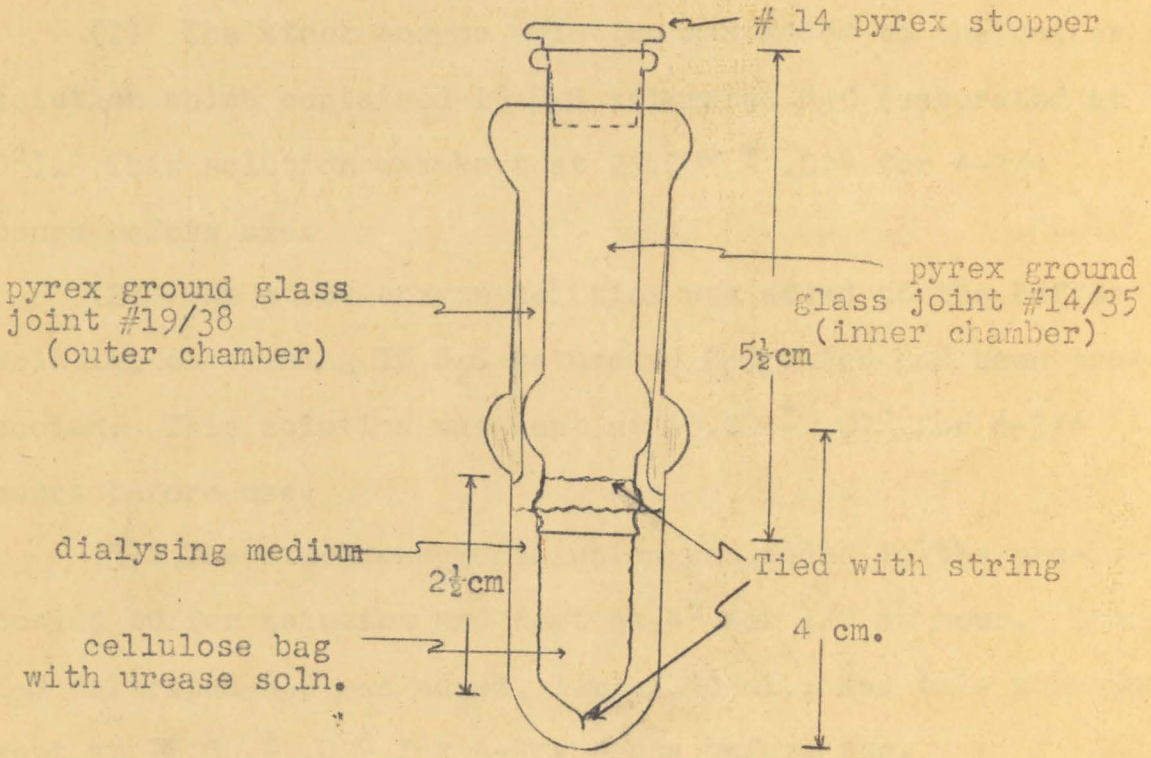


Figure XII

(b) Activation of Enzyme.

The procedure for the determination of the activity was as in Part I, Section 2-c.

The enzyme solution however was prepared in various manners. All the enzyme solutions were prepared so that approximately one ml of solution contained one gamma of protein nitrogen. The buffer concentrations in the enzyme solutions varied with specific investigations, ranging from 0.015 M to 0.3 M in the various buffers under consideration.

Four main procedures were followed for the activation of the enzyme. These were:

(1) The stock enzyme solution was added to the buffer solution which contained 1% H_2S saturated H_2O (saturated at 0^o). This solution was kept at $25.00^o \pm .02^o$ for 4-3/4 hours before use.

(2) The stock enzyme solution was added to the buffer solution containing 1% H_2S saturated H_2O which had been pre-cooled. This solution was kept at $25.00 \pm .02^o$ for 4-3/4 hours before use.

(3) The stock enzyme solution was added to the pre-cooled buffer solution and kept at 4^o for 1/2 an hour. Then H_2S saturated H_2O was added, 1 ml./100 ml., and this solution kept at $25.00 \pm .02^o$ for 4-3/4 hours before use.

(4) The stock enzyme solution was added to the buffer solution (room temperature) and kept at $25.00^o \pm .02^o$ for one hour. Then H_2S saturated H_2O was added, 1 ml./100 ml., and this solution kept at $25.00 \pm .02^o$ for (a) 4 hours, (b) 4-3/4 hours, (c) 5 hours.

Note. The pH of all solutions on final dilution was 7.00 ± 0.02.

c. Discussion of the Methods of Activating the Enzyme

Various methods for effecting the combination of enzyme and co-enzyme were attempted. The evolution of the final and most satisfactory method 4(c) above will be briefly discussed. Evidence was first obtained that one hour's contact between buffer and enzyme at 0° before adding the H_2S was more efficient than one-half hour's contact. This would indicate that the recombination is slow and that H_2S interfers with this. However on completely eliminating H_2S a great decrease in activity is observed. Addition of H_2S to the enzyme before the buffer addition also caused a large decrease in activity. Thus H_2S appears to be necessary for stabilization of the enzyme but also prevents combination of enzyme and co-enzyme. The effect of temperature on the recombination was seen in that a precooled buffer solution on addition to the enzyme caused less activation than did a buffer at room temperature. The final change was made in allowing the enzyme to stand one hour at 25° with the buffer, and then another five hours at 25° after addition of H_2S.

DATA

Table 25

Time Dialyzed versus the Activity of Urease, pH 7.0 and 25°, in 0.015 M Ethylenediamine hydrochloride and 0.15 M sodium-potassium phosphate[1].

Urease # 12A - Dissolved in 0.001 M Cl. ED.[2]
Dialyzed vs. 0.001 M Cl. ED., 450 ml changed daily.
Enzyme preparation: Method (3) (experimental) Buffer concentration as indicated below.
Reaction Mixture: Conc. of urea = 52.90 x 10^{-6} moles/ml.
Conc. of buffer = 0.159 M Cl.ED.

Expt. No.	Conc. of Buffer in enzyme soln.	Time Dialyzed	μmoles NH_3/ml/ 3 min.
VII-4c	0.015 M Cl-ED.	-	12.36
	0.15 M PO_4-K-Na[3]	-	9.76
VII-3a	0.015 M Cl-ED.	3 hrs.	4.88
b	0.15 M PO_4-K-Na	3 hrs	4.60
c	0.015 M Cl-ED	6-3/4 hrs.	3.69[4]
	0.15 M PO_4-K-Na	6-3/4 hrs.	2.18[4]
VII-4a	0.015 M Cl-ED	22 hrs.	13.56[5]
	0.015 M Cl-ED.	22 hrs.	13.42
VII-5a	0.015 M Cl-ED.	48 hrs.	3.07
	0.15 M PO_4-K-Na	48 hrs.	5.59
VII-6a	0.015 M Cl-ED.	72 hrs.	3.82
	0.15 M PO_4-K-Na	72 hrs.	5.94
VII-7a	0.015 M Cl-ED.	92 hrs.	9.31
	0.15 M PO_4-K-Na	92 hrs.	13.53
c	0.015 M Cl-ED.	92 hrs.	10.74[4]
	0.15 M PO_4-K-Na	92 hrs.	15.94[4]
VII-7d	0.15 M PO_4-K-Na	92 hrs.	12.79[6]
VII-8b	0.015 M Cl-ED.	104 hrs.	7.04[7]
	0.15 M PO_4-K-Na	104 hrs.	11.34[7]
8a	0.015 M Cl-ED.	115 hrs.	6.47
	0.15 M PO_4-K-Na	115 hrs.	9.97
	0.015 M Cl-ED	115 hrs.	4.95[8]
VII-9	0.015 M Cl-ED.	7 days	3.74
	0.15 M PO_4-K-Na	7 days	4.90

1. Na_2HPO_4:KH_2PO_4 7:3
2. 0.001 M Cl.ED. = Ethylenediamine hydrochloride
3. 0.15 M PO_4-K-Na = sodium-potassium phosphate as in 1.
4. Enzyme-buffer solution kept one hour at 4° before adding H_2S, then 4-3/4 hours at 25°.
5. 0.02 ml diffusate added to the enzyme solution (4°), before H_2S addition.
6. Reaction mixture buffer - KH_2PO_4-K_2HPO_4.
7. Kept at 25° for only 3-3/4 hours.
8. No H_2S added to the enzyme solution.

Table 26

Time Dialyzed versus the Activity of Urease, at pH 7.0 and 25°, in 0.015 M Ethylenediamine phosphate, 0.015 M Ethylenediamine hydrochloride, 0.3 M Ethylenediamine phosphate.

Urease # 21: Dissolved in 0.001 M Cl-ED.[1]
Dialyzed vs. 0.001 M Cl-ED. - 450 ml changed daily
Enzyme preparation: Method (4)c (see experimental)
Buffer conc. as indicated
Reaction mixture: Urea conc. = 52.90×10^{-6} moles/ml.
Buffer conc. = 0.159 M in the same buffer as enzyme preparation.

Expt. No.	Conc. of Buffer in enzyme soln.	Time Dialyzed	μmoles NH_3/ml./3 min.
VIII-1	0.015 M PO_4-ED[2]	-	3.64[3]
	0.015 M Cl-ED	-	2.10[3]
	0.3 M PO_4-ED	-	3.01[3]
	0.015 M Cl-ED	-	2.34
VIII-2	0.015 M Cl-ED	1 day	3.14
	0.3 M PO_4-ED	1 day	3.60
VIII-4	0.015 M Cl-ED	4 days	2.66
	0.3 M PO_4-ED	4 days	4.07
VIII-5	0.015 M PO_4-ED	7 days	3.29
	0.015 M Cl-ED	7 days	2.47
	0.3 M PO_4-ED	7 days	3.29
	0.015 M PO_4-ED	none	3.48
VIII-6	0.015 M PO_4-ED	13 days	2.67
	0.015 M Cl-ED	13 days	1.56
	0.3 M PO_4-ED	13 days	2.61

1. 0.001 M Cl-ED = 0.001 M ethylenediamine hydrochloride.

2. 0.015 M PO_4-ED = 0.015 M ethylenediamine phosphate.

3. Kept in constant temp. bath at 25.0° for 4 hours instead of 5 hours.

Table 27

Time Dialyzed versus the Activity of Urease, pH 7.0 and 25°, in Ethylenediamine hydrochloride.

Urease B: Dissolved in H_2O: Dialyzed vs. 450 ml H_2O - changed daily
Enzyme preparation - Method (1) see experimental. Buffer 0.015 M Cl-ED[1].
Reaction Mixture: Concentration of urea as specified. Buffer 0.159 M Cl-ED.

Expt. No.	Conc. of urea moles x 10^{-6}ml.	Time Dialyzed	μ Moles NH_3/ml./3 min.
VI-3	52.90	-	5.42
	5.29	-	3.36
VI-4	52.90	1 day	>9.31
	5.29	1 day	5.64
VI-5	52.90	2 days	2.01
	5.29	2 days	0.70

1. 0.015 M Cl-ED = 0.015 M Ethylenediamine hydrochloride.

Table 28

Time Dialyzed versus the Activity of Urease, pH 7.0 and 25°, in 0.015 M Ethylenediamine hydrochloride and 0.15 M Sodium-Potassium Phosphate[1].

Urease #12A: Dissolved in 0.001 M Cl-ED.[2]
Dialyzed vs. 0.001 M Cl-ED. - 2 ml.
Enzyme preparation; Method (3) (see experimental). Buffer as specified below.
Reaction mixture; conc. of urea = 52.90 moles x 10^{-6}/ml.
conc. of buffer = 0.159 M Cl-ED.

Expt. No.	Conc. of Buffer in enzyme soln.	Time dialyzed	Diffusate added to enzyme prep.	μ Moles NH_3/ml./3 min.
VII-4c	0.015 M Cl-ED.	-	-	12.36
	0.15 M PO_4-K-Na[3]	-	-	9.76
VII-4b	0.015 M Cl-ED.	23 hours	-	13.26
	0.15 M PO_4-K-Na	23 hours	-	16.39
VII-5b	0.015 M Cl-ED.	49 hours	-	3.09
	0.015 M Cl-ED.	49 hours	0.02 ml.	2.53
VII-6	0.015 M Cl-ED.	72 hours	-	2.71
	0.015 M Cl-ED.	72 hours	0.02 ml.	2.70
VIII-6[4]	0.015 M PO_4-ED.	5½ months	-	0.372

1. Na_2HPO_4:KH_2PO_4 7:3
2. 0.015 M Cl-ED. = 0.015 M Ethylenediamine hydrochloride
3. 0.15 M PO_4-K-Na = 0.15 M Sodium-potassium phosphate
4. Reaction mixture buffer = 0.159 M Ethylenediamine phosphate.

Table 29

Time Dialyzed versus the Activity of Urease, pH 7.0 and 25°, in 0.17 M Ethylenediamine phosphate, and 0.015 M Ethylenediamine hydrochloride, and 0.15 M Sodium-potassium phosphate[1].

Urease #12A - Dissolved in 0.001 M Cl-ED.[2]
Dialyzed vs. 0.001 M Cl-ED., 450 ml change daily.
Enzyme Preparation: Method 4(b) (see experimental)
Reaction Mixture: conc. of urea = 52.90×10^{-6} moles/ml.
conc. of buffer = 0.159 M Cl-ED.

Expt. No.	Conc. of Buffer in enzyme soln.	Time Dialyzed	μmoles NH_3/ml./3 min.
VII-10a	0.15 M PO_4-K-Na[3]	12 hours	2.98[4]
b	0.015 M Cl-ED.	12 hours	2.58[4]
c	0.15 M PO_4-K-Na	12 hours	5.05[5]
d	0.15 M PO_4-K	12 hours	2.79[5]
e	0.15 M PO_4-Na	12 hours	1.52[6]
VII-11a	0.17 M PO_4-ED.	5 days	10.63
b	0.015 M Cl-ED, 0.05 M KCl & 0.2 M NaCl	5 days	3.34
c	0.015 M Cl-ED	5 days	3.54
d	0.015 M PO_4-K-Na	5 days	4.24
VII-12a	0.17 M PO_4-ED.	6 days	5.52[7]
	0.015 M Cl-ED.	6 days	2.84[7]
VII-13	0.015 M Cl-ED.	7 days	2.99
VII-14a	0.015 M Cl-ED.	14 days	0.897
b	0.3 M Cl-ED	14 days	0.143[8]
g	0.3 M PO_4-ED.	14 days	1.70[8]
VII-15a	0.015 M PO_4-ED.	15 days	1.28[8]
	0.15 M PO_4-ED.	15 days	1.49[8]

1. Na_2HPO_4: KH_2PO_4 7:3
2. 0.001 M Cl-ED. = 0.001 M Ethylenediamine hydrochloride
3. 0.15 M PO_4-K-Na = Sodium-potassium phosphate as in 1.
4. Buffer soln. precooled before adding enzyme, then 1 hour standing at 4° before adding H_2S.
5. Potassium phosphate buffer made as in Part I, Section C.
6. Sodium phosphate buffer made as in Part I, Section C.
7. Enzyme-buffer solution heated to 40° for 1 hour before adding H_2S, then kept at 25° for 4-3/4 hours.
8. Reaction mixture buffer = 0.159 M Ethylenediamine phosphate.

Table 30

Time Dialyzed versus the Activity of Urease, pH 7.0 and 25°, in 0.015 M Ethylenediamine hydrochloride

Urease #13: Dissolved in 0.001 M Cl-ED.[1]
Dialyzed vs. 0.001 M Cl-ED. - 450 ml. changed daily.
Enzyme preparation: Method (4)b (see experimental);
Buffer as indicated below
Reaction mixture: Urea conc. = 52.90×10^{-6} moles/ml.
Buffer conc. = 0.159 M in same buffer as the enzyme.

Expt. No.	Conc. of Buffer in enzyme soln.	Time dialyzed	μ moles NH_3/ml./ 3 min.
VII-18	0.015 M Cl-ED.	-	13.21
	0.015 M Cl-ED.	-	0.456[2]
VII-19	0.015 M Cl-ED.	1 day	15.25
VII-22	0.015 M Cl-ED.	9 days	0.121
	0.015 M PO_4-ED.	9 days	1.028

1. 0.001 M Cl-ED. = 0.001 M Ethylenediamine hydrochloride.

2. H_2S added immediately with the enzyme, then allowed to stand 4-3/4 hours.

Table 31

Effect on Buffer Concentration on the Kinetics of the Urease-Urea System at pH 7.0 and 25° in Ethylenediamine hydrochloride[1].

Experiment VII-13 B

Urease #12A - Dissolved in 0.001 M Cl-ED.[2]
Dialyzed 7 days vs. 0.001 M Cl-ED. 450 ml changed daily
Enzyme preparation: Method 4(b) (see experimental)
Buffer conc. = 0.015 M Cl-ED.
Reaction mixture: Urea conc. = 26.45 x 10^{-6} moles/ml.
Buffer as indicated.

Buffer conc. in Reaction Mix.	$1/S_o$	$1/V$
0.399 M Cl-ED.	0.019	0.673
	0.039	0.752
	0.065	0.950
	0.099	1.09
0.238 M Cl-ED.	0.039	0.843
	0.065	1.02
	0.099	1.12
0.159 M Cl-ED.	0.019	1.00
	0.039	1.02
	0.065	1.29
	0.095	1.54

1. See Figure IX - upper plot.

2. 0.001 M Cl-ED. - 0.001 M ethylenediamine hydrochloride

V = moles x 10^{-6} NH_3/ml./min. S_o = S - V/6 moles x 10^{-6} urea/ml.

Table 32

The Effect of Ethylenediamine hydrochloride Buffer on the Kinetics of the Urease-Urea System at pH 7.0 and 25°.[1]
Experiment V-1

Urease B: Undialyzed, dissolved in H_2O.
Enzyme preparation: Method (1) (see experimental), Buffer conc. = 0.015 M Cl-ED.[2]
Reaction mixture: urea conc. as specified, Buffer Conc.= 0.159 M Cl-ED.

S	$1/S_o$	1/V
26.45	0.038	0.258
15.88	0.063	0.246
10.58	0.094	0.348
8.47	0.118	0.361
7.41	0.135	0.397
6.35	0.158	0.502
5.29	0.189	0.437

S = moles urea x 10^{-6}/ml. $1/S_o$ = 1/ S-V/6 moles urea x 10^{-6}/ml.
1/V = moles x 10^{-6} NH_3/ml/min.

1. See Fig. IX - lower plot.
2. 0.015 M Cl-ED. = 0.015 M ethylenediamine hydrochloride.

Table 33

The Effect of Ethylenediamine Phosphate Buffer on the Kinetics of the Urease-Urea System at pH 7.0 and 25°
Experiment VIII-1

Urease #21 - Dissolved in 0.001 M Cl-ED.[1] (Not dialyzed)
Enzyme preparation: Method 4(a) (see experimental)
Buffer conc. 0.015 M PO_4-ED.[2]
Reaction Mixture: urea conc. as specified, (buffer conc. = 0.159 M PO_4-ED.)

S	$1/S_o$	1/V
52.90	0.019	0.825
26.45	0.0385	1.070
15.88	0.0646	1.268
10.58	0.0974	1.594
5.29	0.1961	2.589

S = moles urea x 10^{-6} moles/ml.; $1/S_o$ = 1/S - V/6 moles urea x 10^{-6}/ml.; 1/V = moles x 10^{-6} NH_3/ml./min.

1. 0.001 M Cl-ED. = 0.001 M ethylenediamine hydrochloride.
2. PO_4-ED. = ethylenediamine phosphate.

Table 34

The Effect of Varying the Ethylenediamine hydrochloride Buffer Concentration on the Rate of the Urease-Catalyzed Hydrolysis of Urea, at pH 7.0 and 25°. Experiment VII-13A[1]

Urease #12A - Dissolved in 0.001 M Cl-ED.[2]
Dialyzed 7 days vs. 0.001 M Cl-ED - 450 ml. changed daily.
Enzyme preparation: Method (4)b. (see experimental), Buffer conc. = 0.015 M Cl-ED,
Reaction mixture: urea conc. = 26.45 x 10^{-6} moles/ml.
Buffer as indicated.

Conc. of Buffer in Reaction Mix.	V = moles x 10^{-6} NH_3/ml./3 min.
0.399 M Cl-ED.	3.99
0.319 M Cl-ED.	3.79
0.238 M Cl-ED.	3.56
0.159 M Cl-ED.	2.94
0.08 M Cl-ED.	0.192

1. Fig. X - lower plot.

2. Cl-ED. = Ethylenediamine hydrochloride.

Table 35

The Effect on Varying the Ethylenediamine Phosphate Buffer Concentration on the Rate of the Urease-Catalyzed Hydrolysis of Urea, at pH 7.0 and 25°. Experiment VII-15.

Urease #12A: Dissolved in 0.001 M Cl-ED[1].
Dialyzed 15 days vs. 0.001 M Cl-ED. - 450 ml. changed daily.
Enzyme preparation: Method (4) b (see experimental)
Buffer conc. = 0.015 M PO_4-ED.[2]
Reaction Mixture: urea conc. = 52.90 x 10^{-6} moles/ml.
Buffer as specified.

Conc. of Buffer in Reaction Mix.	V = moles x 10^{-6} NH_3/ml./3 min.
0.159 M Cl-ED.	1.28
0.106	1.22
0.079	1.78
0.052	0.947
0.026	0.620

1. Cl-ED. = Ethylenediamine hydrochloride.
2. PO_4-ED. = Ethylenediamine phosphate.

Fig. X - upper plot.

Table 36

The Effect of Varying the Ethylenediamine Phosphate Buffer Concentration on the Rate of the Urease-Catalyzed Hydrolysis of Urea, at pH 7.0 and 25°. Experiment VII-22.

Urease #13: Dissolved in 0.001 M Cl-ED.[1]
Dialyzed 9 days vs. 0.001 M Cl-ED. - 450 ml. changed daily
Enzyme Preparation: Method (4) b (see experimental)
Buffer conc. = 0.015 M PO_4-ED.[2]
Reaction Mixture: Urea conc. = 52.90 x 10^{-6} moles/ml.

Conc. of Buffer in Reaction Mix.	V = moles x 10^{-6} NH_3/ml./3 min.
0.159 M PO_4-ED	1.028
0.106 M PO_4-ED	0.664
0.079 M PO_4-ED	0.461
0.052 M PO_4-ED	0.299
0.026 M PO_4-ED	0.227

1. Cl-ED = Ethylenediamine hydrochloride.
2. PO_4-ED = ethylenedaimine phosphate.

See Figure XI - lower plot.

Table 37

The Effect of Varying the Ethylenediamine Phosphate Buffer Concentration on the Rate of the Urease-Catalyzed Hydrolysis of Urea, at pH 7.0 and 25°. Experiment VII-24

Urease #15: Dissolved in 0.001 M Cl-ED.[1]
Dialyzed for 2 days vs. 0.001 M Cl-ED. - 450 ml changed daily
Enzyme Preparation: Method (4) b (see experimental)
Buffer conc. = 0.015 M PO_4-ED.[2]
Reaction Mixture: Urea conc. = 52.90 x 10^{-6} moles/ml.
Buffer as specified.

Conc. of Buffer in Reaction Mix.	V moles x 10^{-6} NH_3/ml./3 min.
0.159 M PO_4-ED.	1.06
0.106	1.46
0.079	1.15
0.052	0.927
0.026	0.721

1. Cl-ED. = Ethylenediamine hydrochloride.
2. PO_4-ED. = Ethylenediamine phosphate.

Table 38

The Effect of Varying the Ethylenediamine Phosphate Buffer Concentration on the Rate of the Urease-Catalyzed Hydrolysis of Urea, at pH 7.0 and 25°. Experiment VIII-6.

Urease #21: Dissolved in 0.001 M Cl-ED.[1]
Dialyzed 13 days vs. 0.001 M Cl-ED.[2] - 450 ml. changed daily.
Enzyme preparation: Method 4 (c) (See experimental)
Buffer conc. = 0.015 M PO_4-ED.
Reaction mix.: Urea conc. = 52.90 x 10^{-6} moles/ml. Buffer as specified.

Conc. of Buffer in Reaction Mix.	V moles x 10^{-6} NH_3/ml./3 min.
0.159 M PO_4-ED.	2.76
0.106 M PO_4-ED.	2.47
0.079 M PO_4-ED.	2.61
0.052 M PO_4-ED.	2.66
0.026 M PO_4-ED.	2.52

1. Cl-ED = Ethylenediamine hydrochloride.
2. PO_4-ED = Ethylenediamine phosphate.

See Figure XI - upper plot.

Table 39

The Effect of Anionic Change in Ethylenediamine Buffers on the Rate of the Urease (dialyzed) - Catalyzed Hydrolysis of Urea, at pH 7.0 and 25°. Experiment VII-22.

Urease #13: Dissolved in 0.001 M Cl-ED.[1]
Dialyzed for 9 days vs. 0.001 M Cl-ED. - 450 ml. changed daily.
Enzyme preparation: Method (4)b (see experimental)
Buffer conc. = 0.3 M in each buffer separately.
Reaction Mixture: Urea conc. = 52.90 x 10^{-6} moles/ml.
Buffer = 0.159 M in each buffer being tested.

Buffer in Enzyme soln. and Reaction mix.	V = moles x 10^{-6} NH_3/ml./3 min.
Citrate - ED.[2]	4.71
Phosphate - ED.	1.13
Maleate - ED.	0.478
Sulphate - ED.	0.422
Chloride - ED.	0.380
Acetate - ED.	0.073

1. Cl-ED. = Ethylenediamine hydrochloride.
2. ED. = Ethylenediamine.

Table 40

The Effect of Anionic Change in Ethylenediamine Buffers on the Rate of the Urease (undialyzed) - Catalyzed Hydrolysis of Urea at pH 7.0 and 25°. Experiment VIII-3

Urease # 21: Dissolved in 0.001 M Cl-ED.[1] Not dialyzed.
Enzyme preparation: Method 4(c) (see experimental)
Buffer conc. = 0.3 M in each buffer separately.
Reaction mixture: Urea conc. = 52.90 x 10^{-6} moles/ml.
Buffer conc. = 0.159 M in each buffer tested.

Buffer in Enzyme Soln. and Reaction Mixture.	V = moles x 10^{-6} NH_3/ml./3 min.
Citrate - ED.[2]	3.86
Phosphate - ED.	3.69
Maleate - ED.	3.57
Sulphate - ED.	3.03
Acetate - ED.	2.66
Chloride - ED.	2.56

1. Cl-ED. = Ethylenediamine hydrochloride.

2. ED = Ethylenediamine.

Table 41

The Effect of Both Anionic and Cationic Changes in the Buffer on the Rate of the Urease Catalyzed Hydrolysis of Urea.

Urease - as specified.
Enzyme preparation: Urease #21, Method 4(c) (see experimental)
Buffer conc. as specified.
Reaction Mixture: Urea conc. = 52.90 x 10^{-6} moles/ml.
Buffer = 0.159 M in the same buffer as enzyme soln.

Expt. No.	Conc. of Buffer in Enzyme soln.	Time Dialyzed	Moles NH_3/ ml./3 min.
VIII-2	0.3 M PO_4-ED.[1]	1 day	3.90
	0.3 M PO_4 THMAM[2]	1 day	3.16
	0.4 M ED-Citrate[3]	1 day	8.69
	0.4 M ED -Acetate[4]	1 day	16.57
VIII-3	0.3 M PO_4-ED.	none	3.69
	0.3 M PO_4-THMAM	none	3.14
VIII-5	0.4 M ED.-Citrate[3]	7 days	7.34
	0.4 M ED.-Acetate[4]	7 days	21.0
	0.3 M PO_4-ED.	7 days	3.29
VIII-6	0.4 M ED.-Citrate[3]	13 days	7.46
	0.4 M ED.-Acetate[4]	13 days	18.0
	0.3 M PO_4-ED.	13 days	2.61

1. Buffer: 0.3 M phosphate and 0.28 M ethylenediamine
2. Buffer: 0.3 M phosphate and 0.59 M Tri(hydroxymethyl) aminomethane.
3. Conc. in this case refers to the ethylenediamine concentration, not citrate. Citrate conc. = 0.27 M.
4. Conc. in this case refers to the ethylenediamine concentration, not acetate. Acetate conc. = 0.67 M.

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PART II

ATTEMPTED PURIFICATION OF THE ADRENOCORTICOTROPIC HORMONE (ACTH)

Attempted Adrenocorticotropic Hormone (ACTH) Purification

Introduction

The impact of cortisone and the adrenocorticotropic hormone (ACTH) in medical practice has thrown much light upon the basic regulatory mechanisms of the body. The use of these substances for the alleviation of such diseases as rheumatoid arthritis and rheumatic fever has resulted in considerable research into the elucidation of these structures.

Of the six hormones identified in the anterior hypophysis, the adrenocorticotropic hormone had been least investigated until 1943 when simultaneously Sayers, White and Long[1] and Li, Evans and Simpson[2] published details of preparation from pig and sheep pituitaries respectively. Earlier Smith[3] had demonstrated that adrenal atrophy following hypophysectomy could be reversed by a factor in the anterior pituitary and many researchers had attempted the isolation of this factor[4].

These protein preparations of Li and Sayers with high adrenocorticotropic potency from pig and sheep glands and later from ox glands[5], appeared to be identical in biological, chemical and physical properties.[1,2,5,6] Their homogeniety was demonstrated by criteria of sedimentation, electrophoresis and constant solubility. Li's protein was shown to have a molecular weight of 20,000, an isoelectric point of pH 4.7 and was water soluble. Its analysis showed

46.35% carbon, 5.89% hydrogen, 15.65% nitrogen and 2.3% sulfur, the latter equating to 7.19% cystine and 1.93% methionine. Biological activity was shown to be lost after precipitation from solution by trichloro-acetic acid, and after digestion by trypsin[2] and papain[7,10]. The biological activity had marked stability to peptic digestion, being unimpaired when 37% was digested, and its thermostability was shown by no loss of activity at neutral pH upon heating for one hundred and twenty minutes in boiling water[8].

Subsequent work on ACTH may be divided into two approaches. First, the investigation of peptic and other hydrolytic agents, initiated by Li[2] and second, the investigation of ultrafiltrates (or dialysable material) from the original pituitary extracts.

Li's original observation that ACTH had marked stability toward peptic digestion indicated that the molecular weight of the active moeity might be much smaller than 20,000. Pepsin and hydrochloric acid digestions[1] produced a series of peptide fragments of lower molecular weight responsible for adrenocorticotropic potency[9,10]. These peptides were not precipitated by trichloroacetic acid and were dialysable.

Further investigation of these peptides by Li[11,12] yielded two values for the average molecular weight, 1200 and 2000. Their average length was computed to be seven to nine amino acid residues. On chromatography of the total hydrolysates of these peptides, the following amino acids

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1. 6 M HCl at 120° for 4 hours; Pepsin in 0.05 M HCl at 37.4° for four to five hours.

were identified: aspartic acid, glutamic acid, lysine, arginine, serine, glycine, threonine, alanine, tyrosine (1.5%), histidine, valine, tryptophane (1.0%), proline, leucines and phenylalanine. Paper partition chromatography on the peptide mixture produced six spots[12] by the ninhydrin test, the immobile spot being the most active. Displacement development analysis on this active spot showed a wide spectrum of materials, with the greatest activity again being in the least mobile component. Carrier displacement analysis on the peptide mixture yielded three major fractions. On the active spot (above) this method indicated five fractions, 60% of the material being inactive and 18% containing activity.

Brink[13] also reported activity of dialysed pepsin digested ACTH, which contained seven to eight amino acids. Li[14] demonstrated that the biological activity could be enhanced of both ACTH and the ACTH peptides by heating a 0.025M hydrochloric acid solution of these in a boiling water bath for thirty minutes.

Concentrates of peptic digestion of ACTH with one hundred to one hundred and fifty times the original activity were obtained by Lesh[15]. These peptides were stated to have a molecular weight between 2,500 - 10,000, and contained twelve amino acids.

Anselmino, Hoffman and Herold[16] claimed to have ultrafiltered some active ACTH through a collodion membrane and Tyslowitz[17] reported ultrafiltration of the hormone through cellophane. About 50% of the solids treated passed through the membrane accompanied by 38% of the biological activity.

Sayers[1] had originally observed that during dialysis a considerable portion of the total activity had been lost. Later Sayers[18] and Munson[19] obtained electrophoretically heterogeneous preparations which were more active than previous homogeneous samples. Cortis-Jones[7] employing Sayers'[1] method of isolation, as modified by Fishman[20], carried out ultrafiltration experiments and showed that 40% of the biological active material will pass through a membrane impermeable to molecules of 13,700 molecular weight but permeable to those of 8,000. Frontal analysis revealed the fact that not more than 10% of the ultrafilterable solids contained activity. The ratio of amino to total nitrogen indicated an average of eight to ten amino acid residues per molecule.

Geschwind[21] obtained "non-protein" fractions by trichloroacetic acid precipitation and dialysis on ACTH as prepared by Li[2]. The trichloroacetic acid supernatant contained from 25% to over 40% of the original activity while 7% to 16% could be dialyzed. These workers[22] also treated sheep pituitary glands with 10% trichloroacetic acid and obtained activity in the "non-protein" supernatant.

Morris[23] reported that the size of the ACTH molecule in solution varies with pH and that this phenomenon appeared to be reversible. Smith[24], however, reported that acid solutions on standing twelve hours, gave different sedimentation values even after neutralization, although no biological activity was lost, indicating a change of molecular size. Therefore the dissociation taking place in acidic

solutions was thought to be irreversible. Smith's[25] investigation on the stability of ACTH solutions showed that on standing nineteen to twenty-four hours the activity is destroyed at pH 7 and 13 in the absence of salts, while sodium chloride and sodium phosphate act as stabilizers. Activity is best retained in acid media.

Specificity of structure work[26] has revealed that both free amino groups and free carboxyl groups are essential. Reduction causing loss of sulphur (1.3% to 0.6% in the peptides) does not effect activity. No SH groups have been found in the peptides and after reduction of the S-S groups of cystine, no biological differences were observed.

As ACTH is known to elaborate the function of the adrenal gland, e.g., production of cortisone, analysis of the ascorbic acid in the adrenal gland is the basis of one method for adrenocorticotropic activity. The ascorbic acid content in the gland is decreased on ACTH injection and this decrease is determined.

The ultimate object of this investigation was the resolution of the mixtures obtained from extraction of horse pituitaries, supplied by G. D. Searle & Co., Chicago. It was hoped that by this means a moeity of particularly high biological activity might be attained so that structure work might be attempted. Three samples were submitted for purification:

XI-191-4b- Release No. 145 (RN 145) - Standard acid acetone extract from horse pituitary. Treated with ammonia and reprecipitated with acetone. Freeze dried from aqueous solution.

XI-191-5 - Release No. 146 (RN 146) - From same preparation as 191-4b but precipitated by 80% acetone in alkaline solution. Freeze dried.

XI-195-1b - Release No. 147 (RN 147) - Prepared by digestion of gland residue from XI-191-4&5. Digestion by pepsin in acid solution followed by standard acid acetone extraction and precipitation.

The majority of the work was directed to sample RN 147. Emphasis was placed on methods which did not call for degradative studies.

The following six methods appeared to offer possibilities for fractional separations and were investigated extensively. 1. Paper Partition Chromatograph, a. Paper Strips, b. Chromatopile. 2. Electrophoresis on Paper. 3. Fractional Precipitation with Picric, Phosphotungstic and Aromatic Sulfonic Acids. 4. Combined Dialysis and Ammonium Sulphate Fractional Precipitation. 5. Dinitrophenylation and Chromatography of the DNP-derivatives. 6. Frontal Analysis.

Each of these methods will be discussed in turn.

Discussion of Results

Section 1. Paper Chromatography

a. Paper Strip Chromatography

The immediate necessity for a means of identification of small amounts of the protein on paper required adaptation of known protein color tests. The ninhydrin test[27] has been widely used in paper chromatography but as shown by de Verdier[28] this test is not satisfactory for polypetides. In the present work the ninhydrin test likewise failed with ACTH.

Three tests were successfully adapted by which one milligram of ACTH could be detected. 1. Folin Ciocalteu[29], 2. Sakaguchi[30] and 3. Pauly[31] tests. (see experimental). The capillary ascending method of paper strip chromatography[32] was employed.

Separations were obtained with the following developers: water, phenol-sodium chloride saturated with water, isobutyric acid saturated with water, and water saturated with methyl-acetate (Table 1).

Using the developers, isobutyric acid saturated with water, phenol-sodium chloride saturated with water, and water, sets of three strips of each sample RN 145, RN 146 and RN 147 were run in each of the three developers, i.e., a total of nine runs for each sample. Three color tests were used. Each strip of a set was tested by one of the three color tests and examined under ultra violet light (Tables 2,3,4). The three color tests were run as it was found that no single color

test indicated all the fractions.

The combined results of the three color tests and ultra violet examination revealed that RN 145 contained three separable fractions under these conditions, RN 146, two fractions with a possible third and RN 147, three fractions with a possible fourth. These were located as follows (Summary Tables 2,3,4):

		R_f values Fraction			
Sample	Developer	1	2	3	4
RN 145	isobutyric a.	1.0-0.8	0.36-0.16	0.15-0.08	
	water	1.0-0.86	0.54-0.4		
	phenol	1.0-0.8	0.57-0.4	0.0	
RN 146	isobutyric a.	1.0-0.72	0.3- 0.03	0.0	
	water	1.0-0.7		0.0	
	phenol	1.0-0.72	0.48-0.37	0.24-0.10	0.0
RN 147	isobutyric a.	1.0-0.72	0.7 -0.5	0.55-0.2	0.1-0.01
	water	1.0-0.67		0.3 -0.0	
	phenol	1.0-0.64	0.61-0.5	0.36-0.21	0.14-0.0

It will be noted (cf. Table 2, experimental) that agreement within a series is not too good. This may be due to the fact that the color tests actually were detecting different parts of the molecule and thus have a wide distribution for any one moeity.

The equivalence of the three samples is expressed below by the summary of R_f values taken from Table 5 (see experimental).

	R_f values Fraction		
	1	2	3
RN 145	1.0-0.88	0.67-0.53	0.0
RN 146	1.0-0.9		0.0
RN 147	1.0-0.8	0.64-0.4	0.13-0.0

Water saturated with methyl acetate was also found to separate sample RN 147 into three components with R_f values of 1) 1.0-0.75, 2) 0.6-0.35, 3) 0.0.

On the basis of these experiments it was thought profitable to use isobutyric acid saturated with water, phenol-sodium chloride saturated with water and water saturated with methyl acetate on a larger scale in the chromatopile.

Work arising from the chromatopile suggested purification of the isobutyric acid and when this was done poor separation was obtained. Consequently possible contaminants of isobutyric acid were tried using strip chromatography, isobutylisobutyrate and isobutyl alcohol, but no separations were obtained.

b. Chromatopile Separations

The chromatopile designed by Mitchell and Haskins[33] was employed to obtain larger quantities of the materials resolved by paper strip chromatography (Section 1a), for biological activity assay.

The three developers isobutyric acid saturated with water, phenol-sodium chloride saturated with water, and water saturated with methyl acetate were used in view of the experiments of section 1a. The zones were identified by the color tests discussed above and by examination in ultra violet light, eluted and lyophilized. In all cases the materials obtained were deliquescent and became gummy brown precipitates on exposure to air. Consequently precipitation from acetone or ammonium sulphate was employed to yield stable dry products.

On developement with isobutyric acid saturated with water, and examination by the Sakaguchi test and ultra violet light, the pile showed two zones. The lyophilized samples were dissolved in acidic solutions and precipitated with four times the volume of acetone. Small quantities were obtained; for example, 4-C-1, 7.8 mg.; 4-C-2, 3.2 mg., from 100 mg. of ACTH (RN-147). Preliminary biological tests[1] showed these two samples to be essentially inactive. It is evident that only a small fraction of the original material was being recovered. On attempting a chromatopile with purified isobutyric acid, far less resolution was obtained.

Developement with phenol-sodium chloride saturated with water produced two zones. However, the pile was not easily freed of phenol and was consequently exceedingly difficult to handle. This approach was therefore abandoned.

The solvent water saturated with methyl acetate produced the greatest resolution. Four zones were eluted and lyophilized. The deliquescent materials so obtained were dissolved in acidic media and precipitated by half-saturated ammonium sulphate. Precipitates C-6-1A, C-6-2A, C-6-3A, and C-6-4A were obtained. The supernatants were then saturated with ammonium sulphate, which produced a precipitate in only one, C-6-4B. These are being tested for adrenocorticotropic activity.

This method would appear to be deficient in many ways. The precipitates recovered from lyophilizing were far more hygroscopic than the original samples. Great losses of

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1. Private communication from Dr. A. Raymond, G. D. Searle & Co., Chicago.

materials occurred (18.1 mg. recovered from 100 mg. of starting material, C-6). This could be due to irreversible adsorption, poor elution, or failure to recover the material on acetone or ammonium sulphate precipitation.

The supernatant of C-6-4B was further investigated as it still had a distinct brown coloration. On dialysis material was found to pass through the membrane, while some material remained in the dialyzing bag. Thus four fractions would seem to be present, 1. dialysable, non precipitable by saturated ammonium sulphate, 2. non-dialysable, non precipitable by saturated ammonium sulphate, 3. precipitable by half-saturated ammonium sulphate and 4. precipitable by saturated ammonium sulphate. These observations led later to the purification by method 3, Combined Dialysis and Ammonium Sulphate Fractional Precipitation.

Section 2

Electrophoresis on Paper

Electrophoresis on paper (paper partition chromatography with applied voltage) as described by Haugaard and Kroner[34] was applied to the RN 147 ACTH sample (Table 6).

The maximum number of components which could be separated was five, at pH 4.0, two of which were slightly superimposed. The five spots were detected by the Pauly color test.

At the isoelectric point, pH 4.7, two spots were visible, slightly on the cathodic side of centre (a. R_f 0.58-0.53, $-R_e$ 0.09-0.03; b. R_f 1.0-0.74, $-R_e$ 0.07-0.0)[1]. The expected R_e value of zero was not obtained. This could result from the

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1. For definition of R_e see Table 6.

paper's not being held perfectly perpendicular to the surface of the liquid. However this is doubtful as the relative position of the spots at this pH fits well within the sequence made with the other pH's.

As the lower pH of 2.0 is approached the lower spot (R_f 0.58-0.53 at pH 4.7) migrates much faster toward the cathode and reaches the edge ($-R_e$ max.). The top spot (R_f 1.0-0.74 at pH 4.7) splits into two portions, one migrating toward the anode ($+R_e$ 0.19-0.1 pH 2.0) while the second remains at the centre (R_e 0.0).

As the higher pH of 7.0 is approached both the upper and lower spots migrate toward the anode. The lower spot approaches the centre line ($-R_e$ 0.09-0.03 at pH 4.7 to $-R_e$ 0.06-0.0). The upper component (R_f 1.0) moves from $-R_e$ 0.07-0.0 to distribute itself evenly across the centre line. A third component presumably a part of the second, splits off at pH 5.0 and moves over to $+R_e$ max. and gains in size with increasing pH.

The five spots at pH 4.0 contain two anomalous $+R_e$ portions, which do not fit into the otherwise smooth flowing pattern.

Thus at pH's above and below the isoelectric point, three major portions are separable (pH 7 and 3 most suitable). A maximum of five fractions are separated at pH 4.0.

Section 3

Fractional Precipitation with Picric, Phosphotungstic and Aromatic Sulfonic Acids

Tyslowitz[17] reported that ultrafiltrates of ACTH obtained on dialysis could be precipitated by phosphotungstic acid and not picric acid. Thus this appeared to offer a means of separation of the mixture of ACTH fractions. A picrate was prepared from RN 147 (Table 7) and on bioassay[1] was reported to have significant activity. Therefore this method was further investigated.

Twelve aromatic sulfonic acids and related compounds were tried as precipitating agents and the supernatants were treated with picric acid. All the picrates melted in the range of 170°-200°. Three groups were selected: 2-naphthol-3,6-disulfonic acid supernatant yielded a picrate (G-3A), m.p. 170°180°; 1-naphthol-4-sulfonic acid supernatant yielded a picrate (G-8A), m.p. 180°-185° and 2-naphthol-6,8-disulfonic acid supernatant yielded a picrate (G-11A), m.p. 185°-190° (Table 7). The first precipitates did not melt below 250°. These three precipitating agents were used on larger quantities of RN 147, yielding initial precipitates G-14, G-15 and G-16. The picrates, G-14A, G-15A and G-16A, did not have the same melting point as in the earlier experiments, but were in the range of 175°-205°. All six precipitates are to be bioassayed.

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1. Private communication - Dr. A. Raymond, G. D. Searle & Co., Chicago.

A third component could be precipitated by the use of phosphotungstic acid. By using 2-naphthol-3, 6-disulfonic acid, picric acid and phosphotungstic acid in this order, three successive precipitates were obtained, G-37, G-37A and G-37B. These are to be bioassayed.

The figures of recovery, given in Table 7, show that not all the materials are being precipitated, (e.g., G-14 and G-14A totaled 59.1 mg from an initial 100 mg.) though perhaps an active fraction may be included.

Many unsuccessful attempts were made to recover the protein from both picric acid and phosphotungstic acid precipitates.

Section 4

Purification by Dialysis and Fractional Ammonium Sulphate Precipitation, on sample RN 147

The isolation of five components was accomplished by a combined dialysis and salting out procedure. The first component, component A, dialyzed through a cellulose bag. Upon lyophilizing this diffusate, a dark hygroscopic residue was left, which became gummy on exposure to air. It is possible that this fraction actually contains two components. One, the smaller molecular weight peptides and the other the compound which is responsible for the hygroscopicity.

The next two components were precipitated by half-saturated ammonium sulphate. On redissolving and dialyzing to remove the salt, one component, component B-a, precipitated

out as a brown precipitate as the pH changed from 4.5 to approximately 7.0, while the other component remained in solution. This precipitation could be due to denaturation, however, no precipitation occurred on dialyzing succeeding fraction. Component, B-a, was recovered by centrifugation, while the dissolved component, component B, was recovered as a white amorphous precipitate on lyophilizing. The fourth component, component C, precipitated out on saturation with ammonium sulphate, while the fifth constituent, D, remained in solution. Component C was removed by centrifugation, redissolved and dialyzed to remove salts. Component D was also dialyzed. Both were recovered as white amorphous precipitates on lyophilizing.

The recovery was excellent, 90-94 mg. from 100 mg. of starting material (Table 8). The amounts recovered decreased from components A to D. The three color tests (p. 118) were positive on four of the fractions (B-a not tested), with fraction A giving the greatest intensity.

Section 5

Dinitrophenylation of ACTH and Chromatography of the Resulting Dinitrophenyl-adrenocorticotropic Hormone (DNP-ACTH)

The use of dinitrophenylfluorobenzene (DNFB) as a tagging agent for the identification of amino acids has been widely applied since Sanger[35] adapted this method to the identification of the end groups in the insulin molecule.

It was thought that this reagent could be used as a test for the homogeniety of "pure" substances and has now been applied to proteins in this work. By use of strongly polar developers on silicic acid-celite columns, protein mixtures can be divided into their dinitrophenyl (DNP) fractions. This method assumes that sufficient excess of DNFB is used so that all the free amino groups of each moeity are attacked. However, in a large molecule this factor might not be too important, as the size of the initial group would outweigh the effect of the dinitrophenyl group.

Dinitrophenylation has been applied to sample RN 147 ACTH and to four of the five precipitates obtained by dialysis and ammonium sulphate precipitation of RN 147, section 4, in order to test the efficiency of the separation by dialysis and ammonium sulphate precipitation and to determine the maximum number of possible constituents.

The sample, RN 147, in solution, was treated with DNFB. Fraction one precipitated out during the reaction. A second fraction was precipitated on addition of hydrochloric acid to the supernatant. The aqueous phase was then extracted with ether, and both the ether phase and the aqueous phase were evaporated to dryness, leaving fractions three and four respectively. These four fractions were benzene extracted and the benzene extracts evaporated to dryness. Chromatography of the benzene insoluble portions of the four fractions divided them into a number of zones.

From the total number of zones observed, it is possible to state that this treatment of sample RN 147 ACTH gives four major zones, each moving with the front of a different developing agent, a fifth zone which moves behind one front and a possible sixth zone which remains on top of the column.

Although DNFB treatment produces four fractions, it is not likely that each of these fractions is composed of different components, but rather it is likely that the four fractions contain similar components in varying proportions. This proportionation is believed due to the mutual solubility of these components in the solvents used for fractionation. This situation is indicated by the chromatographic results.

The first DNP fraction, E-2-A, contained one zone not found in the other three fractions. The third fraction, E-2-C, also contained one zone not found elsewhere. Both the second fraction, E-2-B and the fourth fraction E-2-B-3, were comprised of the same zones (Table 9).

Benzene extraction of the four fractions removed the excess DNFB, dinitrophenol (formed during the reaction) and what appeared to be an unreacted oil, perhaps the constituent causing the hygroscopicity of the earlier fractions (see Section 1). Chromatography of the benzene extracted material, E-2-A-a, E-2-B-a, E-2-C-a, obtained from the three fractions, E-2-A, E-2-B, and E-2-C respectively, gave a maximum of four different zones (Table 10).

Chromatography of the DNP Derivatives of Four of the Five Precipitates obtained by Dialysis and Ammonium Sulphate Precipitation (Section 4, D-3-A, D-3-B, D-3-C, D-1-D).

The procedure for obtaining the precipitates from the reaction of DNFB and the protein RN 147 was altered slightly. The precipitate resulting from the reaction, fraction one, was extracted with ether as was the aqueous phase. The combined ether extracts were evaporated to dryness giving fraction two, and this was further extracted with benzene producing fraction three. The aqueous phase was acidified causing precipitation of fraction four and the aqueous supernatant was evaporated to give fraction five.

A total of five zones were detected in the four precipitates, D-3-A, D-3-B, D-3-C, D-1-D, examined (Table 11). Each precipitate displayed a definite distribution of zones. Each had one main zone and several minor ones. The minor zones in a precipitate however, result from the fact that the original separation into the four precipitates by dialysis and ammonium sulphate was not sharp. For example the minor zones of precipitate D-3-C, result from the presence of some D-3-B and D-1-D in D-3-C.

The first fraction in each case, D-3-A-1, D-3-B-1, D-3-C-1, obtained from D-3-A, D-3-B, and D-3-C, contained the largest amount of material, and also yielded the main zone on chromatography. The quantity of D-1-D available, was so small that after DNFB treatment, no fractionation of products was possible. The reaction mixture was evaporated to

dryness and the residue chromatographed in toto. The second fractions, D-3-B-1-a and D-3-C-1-a, the fourth fractions, D-3-B-2-a and D-3-C-2-a, and the fifth fractions, D-3-B-2-b, and D-3-C-2-b, of the original precipitates contained similar zones, in smaller amounts to those found in fraction one of these precipitates.

The fractions of D-3-A showed a wider distribution of zones, and these fractions, D-3-A-1, D-3-A-1-a, D-3-A-2-a, D-3-A-2-b, contained a zone moving in glacial acetic acid, only found in the benzene extracts, fraction three, of the other precipitates. This zone was dinitrophenol, being carried down by the sticky nature of the original material and consequent precipitates formed from D-3-A.

The chromatographic behavior of each precipitate was as follows:

D-3-A, Main zones moved in 2% MA[1] and 10% MA. Minor zones moved in 2% MA and 25% MA.

D-3-B, Main zonesmoved in 25% MA, Minor zones moved in 10% MA and 50% MA.

D-3-C, Main zones move in 10% MA and 25% MA. Minor zone in 50% MA.

D-1-D, Main zone in 10% MA, Minor zones in 2% and 25% MA.

Thus five of the six zones identified in the initial RN 147 ACTH sample were found in various distributions in the four of the five precipitates obtained by dialysis and ammonium sulphate precipitation. An insufficient amount

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1. See Table 11 for definition.

of the fifth precipitate was on hand to run an analysis and perhaps this contained the sixth zone.

It appears therefore that the efficiency of the dialysis and ammonium sulphate fractional precipitation was not too high. However, a certain degree of separation can be seen by the chromatographic analysis of the DNP precipitates.

Section 6

Frontal Analysis of RN 147 ACTH

Frontal Analysis[1] of 0.05% aqueous solutions were attempted on various adsorbents. Norite A completely adsorbed the protein irreversibly while cellulose[1] and silicic acid-celite (3:1)[2] indicated some separation.

Cellulose adsorption indicated two zones, the first coming through with the first five ml of solvent and the second front following. On lyophilizing the three zones, 1. 0-5 ml, 2. 5-43 ml and 3. the eluate of the column, only significant amounts were found in zone 2. Zones one and two had a refractive index difference of 15×10^{-5}.

Silicic acid-celite adsorption also indicated two zones, with a difference of 22×10^{-5} in refractive index. The first front was through within two ml. However fifteen ml was collected as the zone so that the additional material would act as a carrier for the first front. Three zones were lyophilized, 1. 0-15 ml, 2. 15-46 ml and 3, the eluate of the column. The amounts recovered for future bioassay

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1. See Part III of this thesis.
2. See Section 5, Part II of this thesis.

were, zone 1. 11.0 mg., zone 2. 29.7 mg, zone 3. 1.0 mg, a total of 41.7 mg of the initial 50 mg used.

Though the above separations are not as adequate as might be expected from this method, further work should be carried out, as this approach presents innumerable possibilities.

Section 7

Summary of Results

Paper Strip Chromatography revealed that sample RN 145 contained three fractions, RN 146 two fractions with a possible third and RN 147 three fractions with a possible fourth under the conditions used. The chromatopile isolated two fractions with isobutyric acid as developer and four fractions with water saturated with methyl acetate as developer on sample RN 147.

The electrophoretic work on paper on RN 147 showed the presence of five moeities at pH 4.0 with three sufficiently apart to offer a means of separation above or below the isoelectric point.

Fractional precipitation on sample RN 147 with 2-naphthol-3,6-disulfonic acid, picric acid and phosphotungstic acid produced three fractions as the salts of these acids.

Dialysis and Fractional Precipitation with ammonium sulphate on sample RN 147 isolated five components, three white amorphous precipitates (one very small), one hygroscopic precipitate and one small brown precipitate.

DNP-ACTH (RN 147) chromatography on silicic acid-celite, identified five zones with a possible sixth. The fractions of dialysis and fractional precipitation with ammonium sulphate yielded four zones, with differential grouping in each fraction, showing this fractionation does produce a certain degree of separation.

Frontal analysis of RN 147 indicated two components.

Therefore, it can be stated that RN 147 contains five moeities with a possible sixth.

Fractions to be analysed for adrenocorticotropic activity are summarized in Table 12.

Section 8

Experimental and Data

a. The Adaption of Colorimetric Methods of Identification of Proteins, Peptides and Amino Acids to Paper Chromatography

Three tests have been found to be applicable in identifying the intact ACTH molecule; the Folin-Ciocalteu, the Sakaguchi and the Pauly tests. These will be discussed individually.

1. Folin-Ciocalteu Test[29]

This test was originally developed for the detection of tyrosine and tryptophane. Herriott[36] also found that this test could identify cysteine in the same manner. The phenol reagent was prepared by the method of Folin[29] but used in

a 1:3 dilution[36]. It was found that the concentration of the base used greatly affected the efficiency of the test, in that random concentrations applied to Whatman No. 1 paper caused a high blue background thus obscuring the blue spot of a positive test. The concentration of base giving the lightest background was found to be a solution of 75 gms of potassium carbonate per 100 ml of water.

The procedure is as follows:

The strip is air dried after development. It is then lightly sprayed with the carbonate solution and dried again. The phenol reagent is now sprayed on and a blue color develops immediately. One milligram of ACTH can easily be identified. The sensitivity of the test was examined for three amino acids. Tyrosine can be detected easily in 5 gamma quantities, with 2.5 gamma as the minimum. A positive test is obtained with 5 gamma of tryptophane, and 10 gamma of cysteine.

2. Sakaguchi Test[30]

This method is stated to be specific for arginine, glycocyamine and methyl guanidine. One milligram of ACTH is easily detected as a pink spot by the following procedure:

The 0.02% naphthol solution is mixed in a 1:1 ratio with the 10% sodium hydroxide solution and sprayed on the dry paper after development. The strip is again dried. The chromatogram is then sprayed with sodium hypobromite solution and a reddish color develops immediately. The color fades on standing but can be retained for a longer period of time

if the wet strip is sprinkled with urea. n-Butanol, t-amyl alcohol, collidine, benzyl alcohol, isobutyric acid, methyl acetate and phenol development does not interfere with the test. Five gamma of arginine can be detected, the color remaining a reasonable length of time, while 0.5 gamma produces a color which fades immediately.

3. Pauly Test[31]

This method is used for the identification of histidine but there appears to be no cognizance of the fact that tyrosine works equally well. Using the diazotized solution of sulphanilic acid of Macpherson[31] and coupling in a basic solution, it was found that ACTH produced a yellow color. Here again the composition of the basic medium was found to be important for the production of a light background. It was found that a 1:1 mixture of 20% sodium carbonate and 20% sodium acetate produced the best medium for coupling and giving the lightest background.

The procedure is as follows:

The dried strip is sprayed with the basic medium and dried. Then the strip was sprayed with the cooled diazotized sulphanilic acid. The sprays must be very fine and applied lightly. On final spraying the paper should not appear wet when the color appears. The reagents cause a very light yellow-orange background but there is no difficulty in identifying the intense yellow spot of the positive test. The colors developed for histidine and tyrosine are very characteristic for these two components. Histidine produces a

reddish orange (brick red) color and tyrosine a brownish red. All the other amino acids tested cause the formation of a yellow spot, with the exception of proline and hydroxyproline. These couple producing colorless spots which show up against the yellowish background. Amino acids giving the yellow color are: tryptophane, leucine, isoleucine, cysteine, cystine, phenylalanine, glutamic acid, alanine, aspartic acid, glycine, threonine, serine, valine, arginine, and methionine. The sensitivity of the test was found to be: 0.5 gamma of histidine with 0.25 gamma fairly detectable, 5 gamma tyrosine and tryptophane, and 10 gamma for all the aliphatic amino acids. Tryptophane can be distinguished from the other amino acids at a concentration of above 50 gamma, the spot being yellow brown, while below this quantity it appears the same as the others.

To investigate what portion of the molecule was coupling the following were tested (in test tubes) and the colors obtained were: glycine——yellow, N-acetylglycine——colorless, Tyrosine——deep purple, N-acetyl and N-formyltyrosine——deep purple, O,N-dibenzoyltyrosine——colorless. Thus it can be seen that the aliphatic amino acids are forming diazo-amino compounds while the aromatic are coupling on the ring. Further it can be seen that for tyrosine the hydroxyl group must be free to act as the auxochrome for production of color in the hydroxyazo compound formed.

The Pauly test was not interfered with by development in the following developers: benzyl alcohol, t-amyl alcohol,

collidine iso-butyric acid, n-butanol and methyl acetate. Phenol must be removed from the paper before spraying and this is achieved by extraction[37] without removing the amino acid. The dried phenol developed strip is placed in a soxhlet extractor (volume 100 ml) and 200 ml of 1:1 acetone-ether solution was used as the extracting solvent. The flask with this solution is placed in boiling water. The rate is such that the soxhlet fills in about 2.5-3 minutes. This is run from 15-20 minutes thus filling the soxhlet 5-7 times. The strip is dried and then sprayed as before. In this manner it was found that 5 gamma of histidine could be detected after phenol development and acetone-ether extraction. The Folin test could no doubt be applied after phenol development in this manner.

Reagents

1. Folin test

   a. Phenol reagent phosphotungstic, phosphomolybdic acid[29]

   b. Potassium carbonate solution - 75 gms potassium carbonate per 100 ml of water.

2. Sakaguchi test

   a. 10% sodium hydroxide.

   b. Naphthol 0.02%, made by diluting 20 ml of 0.1% naphthol in 95% alcohol to 100 ml with water.

   c. Sodium hypobromite, 2 grams bromine dissolved in 100 ml of 5% sodium hydroxide. The latter two are kept in dark bottles.

3. Pauly test

a. 1% sulphanilic acid in 10% hydrochloric acid.

b. 5% sodium nitrite solution.

c. 1:1 mixture of 20% sodium carbonate and 20% sodium acetate.

The diazonium solution is made by placing one volume of sulphanilic acid in the sprayer immersed in an ice bath, cooling for 5 minutes, then adding one volume of 5% sodium nitrite. The solution is allowed to stand ten minutes before using.

b. Paper Strip Chromatography - Experimental

Procedure:

The capillary ascent method of Williams and Kirby[32] was used. Whatman No. 1 paper strips 1¼ x 15 inches were employed. The ACTH sample 0.001 gm, dissolved in 0.02 ml of water was applied 2 inches from the bottom of the strip. This was suspended in a one liter test tube, containing the developer in the bottom, by means of a hook through a stopper which closed the test tube. The strip was run until the liquid had traversed 3/4 of its length. It was then removed and air dried. The concentration of ACTH applied varied the R_f value considerably. A 0.002 gm sample per 0.02 ml developed with water had a R_f value of 0.71, while a saturated sample had a value of 0.99.

The ACTH solution could not be kept too long as a bacterial growth soon degraded it and a positive ninhydrin test could be obtained.

c. Chromatopile - experimental

The chromatopile of Mitchell[33] was employed. One hundred milligrams of RN 147 ACTH was dissolved in 10 ml of water in a petri dish and absorbed on 10 sheets of Whatman No. 1, 9 cm filter papers. The dish was wiped dry with an eleventh sheet. Thirty sheets were placed above these and 560 below and the chromatopile assembled. The chromatopile was placed in a large battery jar, containing 50-100 ml of solvent, which was closed by means of paper so an atmosphere saturated with solvent was present. The pile was developed until the liquid had traversed most of the pile (28 hours for isobutyric acid and 10 hours for water saturated with methyl acetate). The pile was then kept moist by enclosing it in a desiccator apparatus. The pile was examined under ultra violet light and every tenth sheet developed by a color test. Zones were assigned by the above tests and then these were eluted with 0.1 N acetic acid, 2 ml per sheet, with a final washing of one-third the original volume. This was done by placing the sheets in a buchner funnel and allowing the liquid to drip through slowly, repeating this three times using suction toward the end. The zones were then lyophilized.

Run 4-C:

Sections based on the Sakaguchi tests, section 4-C-1, sheets 368-450, section 4-C-2, sheets 540-600, were eluted and lyophilized as above. The deliquescent material 4-C-2

was dissolved in 5 ml of 0.1 N acetic acid and 0.3 ml of 6 N hydrochloric acid and then precipitated by addition of four times the volume of acetone, standing six hours at 4°. This was centrifuged and dried in vacuo. Section 4-C-1 was dissolved in 5 ml 0.1 N acetic acid, and was then precipitated by allowing it to stand three hours at 4° after the addition of eight times the volume (40 ml) of acetone. The precipitate was centrifuged and dried in vacuo.

Run 6-C:

The sections, 6-C-1A, sheets 30-150; 6-C-2A, sheets 150-300; 6-C-3A, sheets 300-450; and 6-C-4A, sheets 450-575 based on the Pauly test and ultra violet examination were eluted and lyophilized. The deliquescent precipitates were each dissolved in 2 ml. of 0.1 N acetic acid and 2 ml. of 1 N hydrochloric acid and an equal volume of saturated ammonium sulphate was added. The precipitates were centrifuged and dried in vacuo. The supernatants were saturated with ammonium sulphate; only 6-C-4A produced a precipitate, 6-C-4B. The weights recovered were, 6-C-1A, 2.5 mg; 6-C-2A, 0.8 mg; C-6-3A, 2.3 mg; C-6-4A, 6.9 mg; and C-6-4B, 5.6 mg; a total of 17.6 mg recovered from the original 100 mg.

d. Electrophoresis - Experimental

The experimental procedure of Haugaard[34] was used. Whatman No. 1 sheets 4-3/4 x 23 inches were run through the buffer solutions twice, the excess liquid was pressed out

with a roller and the sheets air dried. Platinum strips (0.003 x 0.253 inches) were woven into the edges of the sheets and 1 mg of RN 147 ACTH dissolved in 0.02 ml of water was applied on the centre 2 inches from the bottom. The sheet was suspended in a battery jar, 6 inches in diameter and 18 inches deep, which contained 250 ml of the developer in the bottom. To seal the glass jar, a glass plate with three holes was used. Two holes were for the electrode clamps and one for a stopper carrying a hook to hold the glass bar on which the paper rested. The apparatus was allowed to stand one-half hour to reach saturation, the paper lowered into the developer and the direct current of 50 volts turned on. Each run lasted from six to eight hours. The paper was removed, dried in air and treated by the Pauly color test.

The following buffers of 1/15 M concentration were used.
pH 2 - 4 made from sodium citrate and hydrochloric acid,
pH 4.7-5 made from acetic acid and sodium acetate,
pH 6 - 7 made from disodium hydrogen phosphate and sodium dihydrogen phosphate.

e. Fractional Precipitation with Picric, Phosphotungstic and Aromatic Sulfonic Acids - Experimental

Experiments G-1 to G-13

To a 0.2 ml. solution containing five mg. of RN 147 ACTH, 0.4 ml. of 0.06 molar precipitating agent was added. This was allowed to stand for one-half an hour and then centrifuged, yielding precipitate G-1 (G-2 etc.). To the

supernatant 0.4 ml. of saturated picric acid solution was added. This precipitate G-1-A was also centrifuged. Both precipitates were dried in vacuo.

Experiments G-14 to 16

One hundred milligrams of RN 147 ACTH was dissolved in 4 ml. of water and 8 ml. of 0.06 M precipitant was added. The precipitate was centrifuged off and 8 ml. of saturated picric acid added to the supernatant. This precipitate was centrifuged down and then both precipitates were dried in vacuo.

Experiment G-37

One hundred milligrams of ACTH RN 147 was dissolved in 4 ml. of water. Eight ml. of 0.06 M 2-naphthol-3,6-disulfonic acid was added and the precipitate G-37 removed by centrifugation. Then 8 ml. of 0.06 M picric acid was added to the supernatant and this precipitate, G-37-A was removed by centrifuging. One drop of 20% phosphotungstic acid was added to the supernatant and again this precipitate was centrifuged to give G-37-B. The three precipitates were dried in vacuo.

The first picric acid sample sent for bioassay # AC-3, was precipitated by adding saturated picric acid to 0.5 ml of 0.1 M acetic acid containing 36.7 mg. of ACTH RN 147. This yielded a 25.5 mg. precipitate.

f. Dialysis and Fractional Precipitation with Ammonium Sulphate. Experimental for Runs D-2, D-3 and D-4

One hundred milligrams of RN 147 ACTH were dissolved in

5 ml. of water and transferred to a cellulose dialyzing bag[1], with two, one ml. washings. The bag was suspended in a 60 ml. Erlenmeyer flask containing 50 ml. of distilled water and rotated slowly for 24 hours at 4°. The 50 ml. dialyzing solution was changed and the bag rotated for another 24 hours. The two 50 ml. portions contained fraction A.

The material in the dialyzing bag was transferred to a 100 ml. centrifuge cup, diluted to 20 ml. and 20 ml. of saturated ammonium sulphate was added. This solution was allowed to stand 24 hours at 4° and the resulting precipitate B, was centrifuged down at 3000r.p.m. The supernatant from B, was saturated with ammonium sulphate and allowed to stand 36 hours. The precipitate B was dissolved in 25 ml. of water and dialyzed to remove salts. After 17 hours a brown precipitate, B-a, had come down and was centrifuged off (the supernatant contained B). Precipitate B-a was dried in vacuo.

The saturated ammonium sulphate solution had yielded a precipitate, C, after standing 36 hours and this was removed by centrifugation. Precipitate C was dissolved in 25 ml. of water and was dialyzed for 24 hours to remove salts as was the supernatant which contained D. All of the above work was done at 4°.

All samples A, B, C and D were lyophilzed. After all the water was removed from A it was left under vacuo for at least 8 hours and then removed immediately on releasing the

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1. Visking Seamless Cellulose Tubing. The Visking Corporation - 6733 West 65th Street, Chicago 38, Illinois.

vacuum. In this manner precipitate A could be completely removed from the flask, otherwise it was sticky and caused difficulty on removal. This brownish white material was very hygroscopic and soon became a sticky brown mass on exposure to air.

All the precipitates with the exception of A and B-a were white amorphous solids.

g. Dinitrophenylation of ACTH and Chromatography of DNP-ACTH - Experimental

The method of Sanger[35] was employed. One hundred milligrams of RN 147 ACTH was dissolved in 2 ml. of water and 100 milligrams of sodium bicarbonate was added. Following this, 0.12 ml. of dinitrofluorobenzene (DNFB) in 2 ml. of ethanol was added. The solution was rapidly agitated with a stirrer for four hours. The precipitate, a brownish yellow material, was then centrifuged and dried in vacuo to give E-2-A. The supernatant was made acidic by addition of 1 ml. 1 N Hydrochloric acid, bringing down E-2-B, a dark yellow precipitate, which was centrifuged and dried in vacuo. The acidic solution stood 12 hours and a fine yellow precipitate formed, E-2-B-2, which was centrifuged and dried as above. The supernatant, E-2-B-3 (water soluble) was extracted with ether to give E-2-C. Both E-2-B-3 and E-2-C were evaporated to dryness in a water bath using an air jet. E-2-C dried to produce a yellow oil and a precipitate and E-2-B-3 formed a yellow sticky precipitate.

All the precipitates except E-2-B-2 were extracted with two 5 ml. portions of benzene, which were then evaporated to dryness yielding, E-2-A-a, E-2-B-a, etc. The original precipitates were cleaned up by this procedure. E-2-B-3 contained mostly sodium chloride. Weights of the precipitates obtained were: E-2-A, 78.1 mg.; E-2-B, 20.1 mg.; E-2-B-2, 5.0 mg,; E-2-C, 3.9 mg.; and E-2-B-3, 22.4 mg.

The benzene solutions (a's) were all gummy yellow brownish precipitates.

Preparation of DNP derivatives of the Fractions Obtained by Dialysis and Fractional Ammonium Sulphate Precipitation

(Section 4)

Fractions used and weights were: D-3-A, 21 mg.; D-3-B, 10 gm.; D-3-C, 7.4 mg.; and D-1-D, 0.3 mg.

The ACTH fraction was dissolved in 0.5 ml. of water and an equal weight of sodium bicarbonate added. DNFB (1 ml/gm. ACTH) in one ml. of ethanol was added. The solution was stirred for two hours. Fraction D-3-A produced a sticky precipitate, D-3-A-1, while fractions D-3-B and D-3-C produced yellow precipitates, D-3-B-1 and D-3-C-1. Fraction D-1-D did not produce a precipitate.

The precipitate in each case was centrifuged and then both precipitate and supernatant were separately extracted with ether. The combined ether solutions were evaporated to dryness to yield a dark orange brittle material (e.g., D-3-A-a). This solid was extracted with benzene, leaving an orange yellow precipitate (e.g., D-3-A-1-a, D-3-B-1-a, etc.) and upon

evaporation of the benzene solution a dark yellow brown oil and a small precipitate (e.g., D-3-B-1-b, etc.) was obtained. The initial supernatant after the ether extraction was acidified with 1 ml. of 1 N hydrochloric acid causing precipitation of a light yellow material (e.g., D-3-A-2-a, etc.). The supernatant was evaporated to dryness leaving mostly sodium chloride with a small yellow precipitate (e.g., D-3-A-2-b, etc.). Fraction D-1-D did not yield a precipitate in any of the above treatments as insufficient starting material was used, so the reaction mixture was evaporated to dryness. The first precipitate from each fraction contained the greatest amount of material.

Chromatographic Procedure for DNP-ACTH Derivatives

The method of Schroeder and Green[1] was used. A 20 cm. No. 1 chromatographic column was used. A 3:1 mixture of Silicic acid (Mallinkrodt special) and Celite No. 545 was poured into the column under slight suction and then the aspirator turned on full (approximately 20 mm. pressure). The prewash was applied: 0.2 volumes[2] ethyl ether, 1.0 volume 1:1 acetone-ether, 0.8 volumes ethyl ether, 1.0 volume ligroin 60-70° and 1.0 volume of developer. The fraction to be investigated was then placed on the column in a suitable solvent (see Tables) and developed with the various developers reported in the data sheets, Tables 9-11.

The DNFB was detected by means of piperidine in the suction flask. Contact between these two reagents produced a yellow color. The DNFB moved through the column first, followed by the dinitrophenol.

1. Private communication from Dr. W. Schroeder and Dr. C. Green, California Institute of Technology.
2. Volume = volume required to wet the column, about 5 ml.

DATA

Table 1

Paper Strip Chromatography on RN 147 ACTH

0.001 gm. per run

Developer	Time Run in Hours	Zone	R_f Values[1] Folin	Color test Sakaguchi	Pauly
Phenol/water[2]	20	1		1.0-0.89	
Phenol/water	43	1	1.0-0.7		
		2	0.0		
Water	8	1	0.99-0.75		
		2	0.67-0.58		
Benzyl alcohol	43	1	0.64-0.55		
		2	0.0		
Isobutyric acid	27	1	1.0-0.63	1.0-0.57	1.0-0.52
		2	0.58-0.42	0.42	0.57-0.4
Water/methyl acetate[3]	6	1	1.0-0.75	1.0-0.8	1.0-0.75
		2	0.65-0.5		0.65-0.52
		3		0.0	0.0
Water/methyl acetate[3]	7½	1			1.0-0.84
		2			0.68-0.57
Water/ethyl acetate[4]	7½	1			0.81-0.71
		2			0.52-0.43

No movement in: n-butanol/water; collidine/water; t-amyl-alcohol/water; 95% ethanol; methyl alcohol-methyl acetate/water; ethylacetate/water; methyl acetate/water; isobutyl alcohol/water and isobutylisobutyrate/water.

1. R_f value is measured from the top of the spot to the bottom of the strip over the distance the solvent has moved.
2. Phenol saturated with water.
3. Water saturated with methyl acetate.
4. Water saturated with ethyl acetate.

R_f Values for Paper Strip Chromatography on Samples RN 145, RN 146
ACTH 0.001 gm. per run

Table 2 - RN 145

Developer	Zone	Folin	U.V.[1] Folin	Color Test Pauly	U.V.[1] Pauly	Sakaguchi	U.V.[1] Sakaguchi
water	1	0.48-0.38		0.99-0.86	0.99-0.86	1.0-0.86	1.0-0.86
isobutyric acid/ water	1	1.0 -0.88	1.0 -0.88		0.99-0.5		
	2		0.36-0.16	0.23-0.08	0.23-0.08	0.15-0.08	
phenol-NaCl/ water[2]	1	1.0 -0.80	1.0 -0.80	1.0 -0.73	1.0 -0.73	1.0 -0.80	
	2		0.47		0.53-0.40	0.57-0.43	
	3		0.0	0.0	0.0		

Table 3 - RN 146

Developer	Zone	Folin	U.V.[1] Folin	Color Test Pauly	U.V.[1] Pauly	Sakaguchi	U.V.[1] Sakaguchi
Water	1	0.73		1.0 - 0.73	1.0 -0.73	1.0 - 0.72	
	2			0.0	0.0	0.0	0.0
isobutyric acid/ water	1	0.99-0.87			1.0 -0.72	1.0 -0.69	1.0 -0.69
	2		0.23-0.07	0.28-0.03	0.28-0.03		
	3				0.0		
phenol-NaCl/ water[2]	1		1.0 - 0.72			1.0 -0.74	1.0 -0.71
	2	0.48-0.39	0.48-0.39				
	3	0.24-0.10	0.24-0.10			0.0	0.21-0.0

1. Ultra violet light examination of color test.
2. Phenol saturated with water and sodium chloride.

Table 4

R_f values for Paper Strip Chromatography of Sample RN 147 ACTH 0.001 gm. per run

Developer	Zone	Folin	U.V[1] Folin	Pauly	U.V[1] Pauly	Sakaguchi	U.V[1] Sakaguchi
water	1	0.85-0.67	1.0 -0.65	1.0 -0.75	1.0 -0.75	1.0 -0.81	
	2	0.35-0.27	0.25-0.0	0.3 -0.0	0.3 -0.0	0.0	
isobutyric acid/ water	1			0.98-0.47	1.0 -0.47	0.98-0.72	
	2		0.7 -0.6	0.72-0.57	0.72-0.57		
	3	0.55-0.41	0.3 -0.2			0.29-0.22	
	4	0.07-0.01	0.07-0.01	0.12-0.04	0.10-0.0	0.14-0.07	
phenol-NaCl/ water[2]	1	1.0 -0.64	1.0 -0.64			0.1 00.59	1.0 -0.59
	2	0.61-0.5	0.61-0.5			0.45-0.3	0.2 -0.15
	3	0.36-0.21	0.36-0.21				
	4	0.14-0.07	0.0			0.0	0.0

1. Ultra violet examination of the color test.
2. Phenol saturated with water and sodium chloride.

Table 5

R_f values for Paper Strip Chromatography of Samples RN 145, RN 146 and RN 147 ACTH Run Simultaneously

		Isobutyric Acid Dev.		Water Developed		Phenol Developed	
Sample	Zone	Folin	U.V[1] Folin	Pauly	U.V[1] Pauly	Sakaguchi	U.V[1] Sakaguchi
RN 145	1	1.0 -0.8	1.0 -0.8	1.0 -0.88	1.0 -0.88		
	2	0.67-0.53					
	3		0.0	0.0	0.0		
RN 146	1	1.0 -0.9	1.0 -0.9	1.0 -0.96	1.0 -0.96	1.0 -0.68	1.0 -0.68
	2		0.0	0.0	0.88-0.8	0.0	0.0
RN 147	1	1.0 -0.9	1.0 -0.9	1.0 -0.8	1.0 -0.8	1.0 -0.68	1.0 -0.68
	2	0.5	0.6 -0.4				
	3	0.13-0.10	0.13-0.10	0.3 -0.0	0.0	0.0	0.0

1. Ultra violet examination of the color test.

Table 6

Electrophoretic Behavior of RN 147 ACTH on Paper with Varying pH

pH	Zone No.	R_f	$-R_e$ [1]	$+R_e$ [2]
7.0	1	0.56-0.42	0.06-0.0	
	2	1.0 -0.83	0	0
	3	1.0		max-0.03 [3]
6.0	1	1.0 -0.72	0.06-0.0	
	2	0.67-0.58	0.07-0.0	
	3	1.0 -0.83		max-0.07
5.0	1	0.6 -0.53	0.03-0.0	
	2	0.93-0.73	0.08-0.03	
	3	1.0 -0.8		max-0.2
4.7	1	0.58-0.53	0.09-0.03	
	2	1.00-0.74	0.07-0.0	
4.0	1	1.0 -0.86	0.08-0.0	
	2	0.78-0.74	0.10-0.07	
	3	0.91-0.81	0	0
	4	1.0 -0.81		max-0.17
	5	0.98-0.88		0.17-0.12
3.0	1	1.0 -0.83	0.11-0.06	
	2	1.0 -0.89	0.08	
	3	0.58-0.53	0.07-0.01	
2.0	1	1.0 -0.78	0	0
	2	1.0 -0.92		0.19-0.1

Voltage used was 50 DC. Solvent used was water saturated with methyl acetate. Time of each run was between 6-7 hours. Zones were identified by means of the Pauly test.

1. R_e = $\frac{\text{distance zone is from the centre line}}{\text{distance solvent front has moved}}$
   $-R_e$ = R_e toward the cathode
2. $+R_e$ = R_e toward the anode

3. Max = the spot had moved to the edge of the paper.

Table 7

Fractional Precipitation with Picric, Phosphotungstic and Aromatic Sulfonic Acids using RN 147 ACTH

Exp't No.	First precipitant[1]	Weight of[2] first ppt. x 10^{-3} gm.	Weight of[3] second ppt. x 10^{-3} gm.	m.p. of second ppt.
G-1	2,4-dinitro-1-naphthol-7 sulfonic acid	4.9	-	
G-3	2-naphthol-3,6-disulfonic acid (Na salt)	1.1	1.4	170°-180°
G-6	2-naphthol-8-sulfonic acid	1.9	1.0	180°-185°
G-7	2,5 dichlorobenzene-sulfonic acid	1.6	1.3	182°-187°
G-8	1-naphthol-4-sulfonic acid	2.1	2.3	180°-185°
G-9	2-naphthol-6-sulfonic acid	1.7	2.3	182°-197°
G-10	1-naphthol-5-sulfonic acid	2.8	1.2	185°-190°
G-11	2-naphthol-6,8-disulfonic acid (Na salt)	1.6	1.4	185°-190°
G-12	1-nitroanthraquinone-8-sulfonic acid	4.3	-	
G-13	β-naphthoquinone-4-sulfonic acid (Na salt)	1.7	1.7	190°-195°
G-14[4]	2-naphthol-3,6-disulfonic acid	27.7	37.4	198°-205°
G-15[4]	1-naphthol-4-sulfonic acid	28.6	36.4	175°-183°
G-16[4]	2-naphthol-6,8-disulfonic acid (Na salt)	32.6	20.4	188°-193°
G-37	2-naphthol-3,6-disulfonic acid (Na salt)	192.2	537.7	
G-37B[5]	Phosphotungstic acid	87.8		
AC-3[6]	picric acid	25.4		

1. With five milligrams of RN 147 ACTH.
2. These did not melt below 250°.
3. The supernatant from the first precipitate (e.g. G-1A, from G-1) was treated with picric acid.
4. With 100 mg. of RN 147 ACTH.
5. The supernatant from the picric acid precipitation was treated with phosphotungstic acid.
6. With 36.7 mg. RN 147 ACTH.

Table 8

Weights of Fractions Recovered By Dialysis and Ammonium Sulphate Fractional Precipitation
100 mg. per run

Fraction	Weight	Fraction	Weight
D-2-A	33.0 mg.	D-3-A	40.0 mg.
D-2-B[1]	44.3	D-3-B-a[2]	7.1
D-2-C	9.8	D-3-B	31.9
D-2-D	6.9	D-3-C	10.8
	94.0	D-3-D	1.7
			91.5

D-4-A	52.0 mg.
D-4-B-a[2]	3.5
D-4-B	20.3
D-4-C	12.4
D-4-D	negligible
	88.2

1. Solid material which had precipitated on dialyzing was not removed before lyophilizing.
2. Solid material which came down on dialyzing precipitate B.

Table 9

Chromatography of DNP-ACTH RN 147 (Run E-2)

Reading from left to right are the different zones as they appeared on development.

	Developers					
Fraction[1]	2% AA-B[2]	2%MA[3]	10%MA[3]	25%MA[3]	50%MA[3]	Top of Column
E-2-A	a	b	c	d		f
E-2-B	a	b	c	d	e	
E-2-C[1]		b				
E-2-C-1[4]	a	b,g[5]				
E-2-B-3	a	b	c	d	e	

1. All fractions were dissolved in glacial acetic acid except E-2-C, where 2% acetic acid in benzene was used.
2. 2% acetic acid in benzene.
3. %MA = % of water (saturated with methyl acetate) in glacial acetic acid.
4. The material not soluble in 2% acetic acid in benzene from E-2-C, dissolved in glacial acetic acid.
5. This is a second front in this developer.

Note: all zones moved with the front, except g.

Table 10

Chromatography of the Benzene Extracts of the DNP-ACTH RN 147 of Table 9.

Reading from left to right are the different zones as they appeared on development.

	Developers		
Fraction[1]	Methylene chloride	2% AA-B[2]	Top of Column
E-2-A-a[3]	a[4], b	c,d	e
E-2-B-a[3]	a, b[5]	c	e
E-2-B-a	omitted	a,b,c	
E-2-C-a[3]	a, b[5]	c	

1. The fractions were dissolved in methylene chloride.
2. 2% acetic acid in benzene.
3. Dinitrofluorobenzene came through first.
4. Zone a was dinitrophenol, moving behind the DNFB.
5. The majority of the fraction.

Table 11

Chromatography of the DNP-Derivatives of the Precipitates Obtained by Dialysis and Ammonium Sulphate Precipitation

Reading from left to right are the different zones as they appeared on development.

	Developers					
Fraction[1]	G-AA[2]	2%AA-B[3]	2%MA[4]	10%MA[4]	25%MA[4]	50%MA[4]
D-3-A-1	a		c,d	e[5]	g	
D-3-A-1-a	a		c[5],d	e		
D-3-A-1-b	a					
D-3-A-2-a	a	b	c,d[5]			
D-3-A-2-b	a		c	e,f		
D-3-B-1				e[6]	g[5]	h[6]
D-3-B-1-a[7]						
D-3-B-1-b[8]	a	b				
D-3-B-2-a				e	g[5]	h[6]
D-3-B-2-b					g	
D-3-C-1				e[5]	g[5]	h[6]
D-3-C-1-a				e[5]	g	
D-3-C-1-b	a					
D-3-C-2-a				e[5]	g[5]	
D-3-C-2-b				e	g	
D-1-D-1			c,d	e[5]	g	

1. Each fraction dissolved in glacial acetic acid.
2. Glacial acetic acid.
3. 2% acetic acid in benzene.
4. %MA = % water (saturated with methyl acetate) in glacial acetic acid.
5. Majority of the fraction.
6. Only a trace present.
7. Not sufficient on hand to run.
8. Fraction dissolved in 2% glacial acetic acid in benzene.

Table 12

Samples submitted for Bioassay

1. Fractional Precipitation by Picric, Phosphotungstic, and aromatic Sulfonic Acids.

G-14	17.4 mg.	G-14A	33.8 mg.	G-37	16.2 mg.
G-15	27.4	G-15A	45.3	G-37A	40.3
G-16	32.4	G-16A	18.3	G-37B	135.7

AC-3 25.4 mg.

2. Chromatopile Fractions

4-C-1	7.8 mg.	6-C-1A	2.5 mg.
4-C-2	3.2	6-C-2A	0.8
		6-C-3A	2.3
		6-C-4A	6.9
		6-C-4B	5.6

3. Purification by Dialysis and Fractional Ammonium Sulphate Precipitation

D-4-A	52.0 mg.
D-4-B-a	3.5
D-4-B	20.0
D-4-C	12.4
D-3-D	1.7

4. Purification by Frontal Analysis

Cellulose FA. LV		Zone 2	37.2 mg.
Silicic acid:			
Celite	FA. LVI	Zone 1	11.0
		Zone 2	29.7
		Zone 3	1.0

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PART III

INSTRUMENTAL ADSORPTION ANALYSIS

Instrumental Adsorption Analysis

Introduction

Chromatographic analysis has undergone extensive development since 1906 until today it is an indispensable tool for research in many branches of biology and chemistry. One of the greatest steps in the instrumentation of this method has been carried out by Tiselius[1-5] and co-workers since 1940.

The basic principle behind the Tiselius apparatus for adsorption analysis of colorless substances is the flowing or liquid chromatogram, in which the concentration of the solute leaving the adsorbent is determined continuously by refractive index measurements. In the earliest apparatus[1-4] the solution after passing the filter was forced up into a cuvette where layers of solution with different composition were formed. The changes in concentration were observed as in electrophoresis with the Philpot-Svensson slit method. The difficulty of obtaining layers with small differences of specific gravity caused this machine to be abandoned in favor of one where the concentration was determined in a very small cuvette as the liquid emerged from the filter[5]. This procedure has several advantages over older chromatographic methods. It is especially suited for colorless substances and is independent of the color of the adsorbent. The separation of the different components is much greater in the solution leaving the filter than in the column which means that the selectivity increases considerably.

And finally the procedure is especially suited for quantitative evaluations of the adsorption phenomena in the column. By means of an interferometer arrangement the concentration changes are determined by measuring the refractive index to within 1×10^{-6} refractive index units. A self recording instrument was designed by Claesson[6] with an accuracy of 3×10^{-5} in refractive index and 0.3 ml. in volume.

Adaption for larger amounts of material was made[7] by use of coupled filters with diminishing diameters. The next improvement on the instrument was made by Hagdahl[8]. A small mixer was placed between the filter and the cuvette. This prevented layering of the solution as it entered the cuvette, the layering giving rise to interference fringes. This mixer was adapted for coupled filters[9] which brought about a concentration of the solute and so prevented blurring and smearing of the front.

The apparatus to date has four different experimental arrangements: 1. Frontal analysis, 2. Elution analysis, 3. Displacement analysis, and 4. Carrier Displacement analysis.

1. Frontal analysis[1-5]

This is the simplest procedure. The solution to be analyzed is forced through the filter filled with adsorbent. The solute is adsorbed and moves forward with a sharp front as the filter becomes saturated. The refractive index of the solution leaving the filter is measured and plotted

against the corresponding total volume that has passed the filter. The resulting plots are step wise, each step indicating a new component. The volume that has passed before a certain step is reached is called the retention volume. The retention volume of a substance is larger the greater the adsorption but increases also with increasing dilution of the solute. Only the least adsorbed component is obtained pure.

2. Elution analysis[1-5]

This procedure is identical with chromatographic analysis. A small amount of the mixture to be analysed is adsorbed on the top of the filter filled with adsorbent and then eluted with a suitable solvent. Thus the components are made to move as separate zones through the column. The curve obtained when plotting refractive index versus volume shows peaks, each peak indicating a pure component, the area of the peak being proportional to the amount of the component present. On account of tailing of peaks and irreversible adsorption this method often gives unsatisfactory results.

3. Displacement analysis[10]

The disadvantages of elution analysis are overcome by this method. As in elution analysis a small volume of solution is forced into the column, then a solution of a solute, the developer, that is more strongly adsorbed than any one of the components of the mixture is forced through the column. The components are now displaced by this solute and also

displace one another. The plots from this type are step wise, each step indicating only one component. For a given concentration of developer the step for a single component always has the same height and its length is proportional to the amount of the component present. Both qualitative and quantitative compositions of the solution are immediately given. One drawback to this elegant method is that irreversible adsorption may cause loss of material.

4. Carrier Displacement analysis[11]

The disadvantage of displacement analysis, i.e., that the zones are in close contact, is overcome in carrier displacement. This is accomplished by interposing a number of substances of intermediate adsorption affinity called "carriers". In displacement chromatography substances appear in order of their adsorption affinities, independent of their concentrations. Therefore a given substance will always appear against one definite boundary. If a series of carriers is properly selected the components of the sample arrange themselves between broad zones of carrier substances. Thus the components are separated and recovered. Stepwise curves are obtained with the components concentrated between steps.

Theoretical considerations of these methods have been carefully studied. The calculation of the quantitative composition of a solution from an adsorption column has been derived by means of the Langmuir adsorption isotherm[6,10,12,13].

Applications of the above methods.

Tiselius' original purpose in designing the instrument, to fractionate the constituents of bacteria and breakdown products of proteins, has seen many ramifications. Carbohydrates, fatty acids, amino acids, peptides and proteins have been widely investigated.

Frontal Analysis

Glucose, lactose[2], and sucrose[14] are the only sugars which have been chromatographed in this manner. Fatty acid separation has received much attention. Claesson[4,6,15] has extensively studied separations of homologous series of C_6-C_{20} saturated, unsaturated, and branched fatty acids, their ethyl esters, alcohols and dibasic aliphatic acids in ethanol. The lower fatty acids have also been successfully separated[16]. Amino acids and peptides have been chromatographed by Tiselius and co-workers[3,4,17,18]. Egg albumin[19] and its peptic hydrolysis products were satisfactorily handled.

Elution analysis

This method has not been widely used. Fatty acids[6] have been eluted, amino acids, peptides[17] and egg albumin[17] have also been studied in this manner.

Displacement analysis

Adsorption analysis in this form has been extensively investigated. Carbohydrates have been analysed using phenol as a developer[7,10]. Tiselius[10,19,20] has separated mono to hexa polysaccharides. Hagdahl and Holman[16,22] have displaced fatty acids by their higher homologues. It was

observed that increased separability of fatty acids was produced by depressed solubility in the solvents used, with picric acid being used as the developer. Separation of C_1-C_{22}[23] fatty acids was accomplished. Amino acids and peptides have also been analyzed[15,17]. Li[24] developed ACTH peptides with zephiran chloride by this method. Analysis of gases and vapours[6] by displacement analysis has been applied to hydrocarbons.

Carrier Displacement analysis

Three investigations using this method are reported. Tiselius[11] separated amino acids and dipeptides in homologous alcohol mixtures. Holman[25] applied this method to fatty acids in fatty acid esters and also to the separation of alkyl halides, alcohols and esters. Li[24] used this method for the resolution of ACTH peptides.

The report herein deals with the adsorption analysis machine built by the Chemistry Instrument Shop, California Institute of Technology, and its description, operational mechanics, and applications.

Section 1 Description of Apparatus

a. Acknowledgements

This instrument was designed and built by the Chemistry Instrument Shop, California Institute of Technology, in particular by Mr. Arnold Wilmott and Mr. R. J. Penfold, with technical advice from Dr. S. Swingle, Dr. S. J. Singer, Professor R. B. Corey and Professor J. H. Sturdivant. Dr. Stig Claesson contributed helpful suggestions when the construction was under discussion.

The determination of refractive index was selected as the means to follow changes in concentration. The sensitivity of the instrument is such that changes of 2×10^{-5} in refractive index can be recorded.

b. Optical System

The optical system is shown schematically in Figure I. The light source S is a mercury arc. The light beam passes the filter F, a Gaertner green, L-541 E, and emerges through the slit S_1 to fall on the lens L_1, a Bausch and Lomb DC x 5-050 f. 200 mm. This lens focuses the light on the cross hair C, between the adjustable knife edges K. The lens L_2, a Bausch and Lomb DC x 5-030, f. 33 mm., then forms an image of the cross hair in front of the eyepiece E, which is attached to the slide S_4. On leaving the lens L_2 the beam enters the solvent cell S_2, passes through the prism P, containing the solution to be analysed, and passes out through the second solvent cell S_3. Glass windows W enclose the

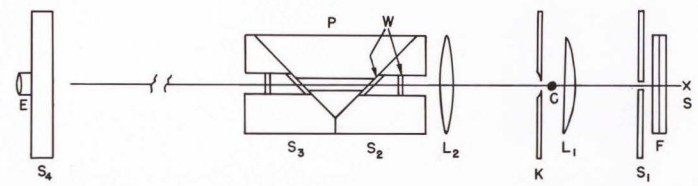


Figure I

Schematic Diagram of the
Optical System

solvent cells and prism. The beam when passing through the 90° prism P, filled with the same solvent as in the solvent cell will emerge in the same plane as the direction of the incoming beam. However, if the refractive index of the solution in the prism is higher than that in the solvent cells, due to dissolved materials, the beam emerges from the prism deflected upwards. This upward deflection is followed by means of the eyepiece E on the movable micrometer slide S_4. The cross hair is kept in the centre of the eyepiece and its motion is read on the slide. This slide reading can be reproduced to within ± 0.003 units, which represents a change in refractive index of ± 2×10^{-5}. The eyepiece has a 3.5 magnification and is fixed on a Gaertner Micrometer slide, M-342.

c. Prism and Solvent Cells

A 90° prism, Figure II, is held at a 45° angle to the incoming beam. On either side the solvent cells rest against the prism. The solvent cell is closed at the outer end by means of a glass window held in place by a screw flange nut, and the inner end, the 45° face, by a glass window which is countersunk and is forced against the prism by a screw arrangement. The light beam enters the first window at 90°, passes through the solvent, then the glass window at 45° and emerges into the prism parallel to the original beam. The amount by which the beam is deflected upwards in the prism depends on the concentration of the solute in the solution through which the beam is passing.

The prism has a channel of 1/8 inch bore parallel to the

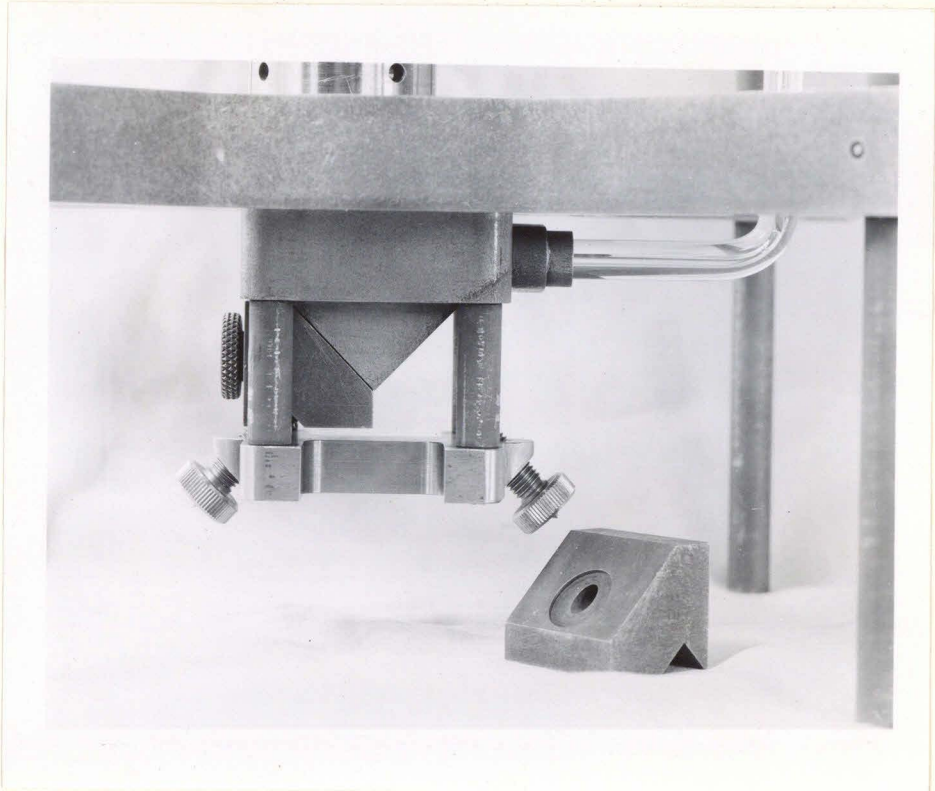


Figure II

Prism and Solvent Cells
Assembly

beam; a 7/16 inch long by 1/32 inch diameter inlet entering flush with the edge of the prism feeding onto the window and a 1/16 inch diameter outlet on the other end of the channel. The outlet has a larger bore than the inlet to prevent any back pressure. The outlet leads to a capillary tube, 1.25-1.75 mm. inner diameter. The windows are made from carefully selected microscope slides.

The solution on leaving the capillary tube is collected in a vessel to measure the volume. A photo-electric drop counter will be installed later.

The prism and solvent cells are supported in a frame assembly, (cf. Figures III and IV) which sits in a water-bath. This assembly is supported on V blocks to assure proper alignment. The water bath is likewise supported on V blocks. The water bath window holders are constructed in one piece so as to remain parallel under all environmental variations.

The whole apparatus is assembled on a channel iron frame, Figure V, for rigidity. The sensitivity is so fine that it was found necessary to place five inch paper tubes between the eyepiece and the bath, and between the bath and the cross hair, to cut down all convection currents as much as possible.

d. Filters for the Adsorbent and Syringes (Solution Containers)

Two complete assemblies were constructed. One a "Preparative Adsorption Assembly" to be used for large quanities and a smaller "Analytical Adsorption Assembly" for more

Figure III

Preparative Adsorption
Assembly

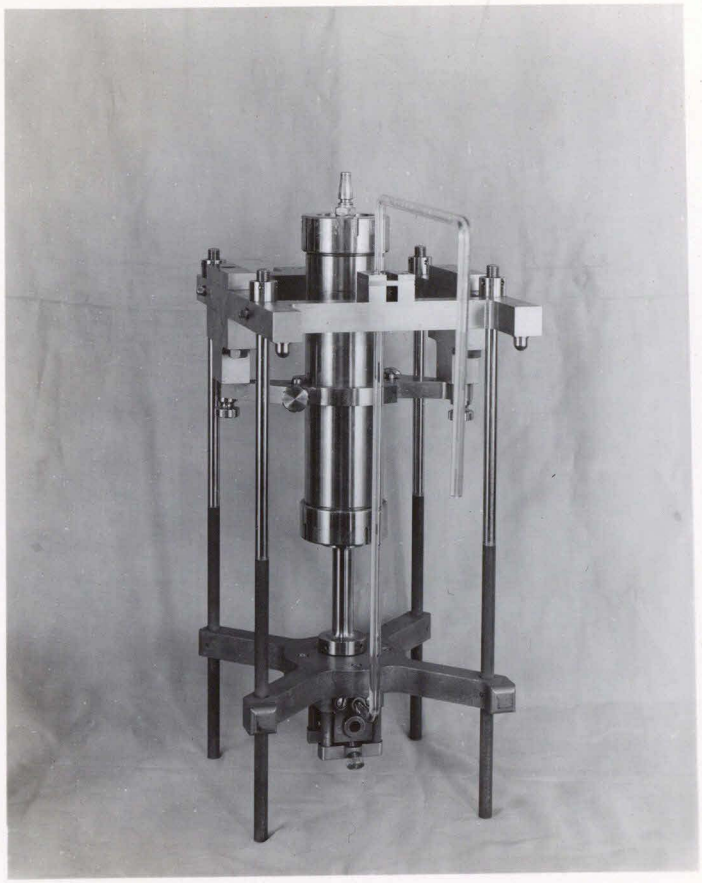


Figure IV

Analytical Adsorption
Assembly

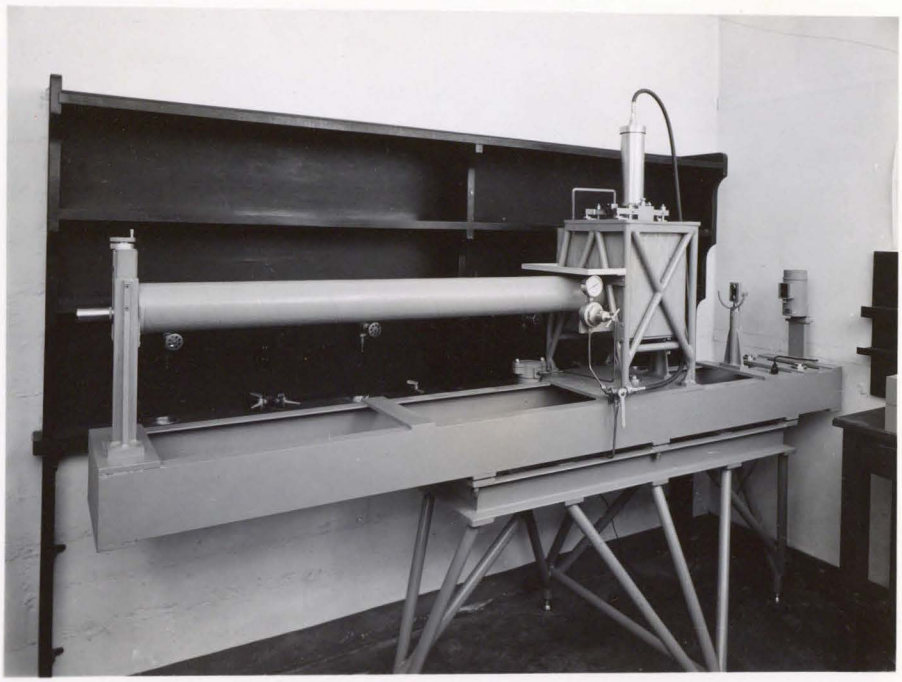


Figure V

Complete Assembly for
Instrumental Adsorption Analysis

selective chromatography.

Preparative Adsorption Assembly - Figure III.

This assembly is used as a coupled filter apparatus.

Filters - Figure VI

The filters consist of a cylindrical metal tubes containing the adsorbent between filter papers supported by perforated discs. One filter is constructed to fit into the top of the prism while the others are coupled on top of this.

The prism filter is threaded at both ends. The lower end screws into the prism socket which leads to the prism inlet channel. The upper end screws into an adapter plate which in turn holds the next filter. The prism filter has a 7/8 inch long by 3/4 inch diameter adsorption section. A perforated disc sits above the exit channel, which is 3/16 inch long by 1/16 inch diameter which could be packed as a mixer if desired. The upper perforated disc sits in a recess in the filter.

Two adapter plates are provided, one fitting three filters and the second is for the largest filter. The adapter plates are held onto the filters by means of six screws. The upper faces are countersunk and hold a perforated disc which acts as a base for the next filter.

The sizes of the four filters which couple to these plates are; 4 inches long by 2 inches in diameter; 2 inches long by 1½ inches in diameter; 2 inches long by 3/4 inch in diameter; and 1 inch long by 3/4 inch in diameter.

The upper ends of these filters have large flanges made to fit the syringe which carries the solution. Perforated

Figure VI

Filters and Syringe Apparatus
for the Preparative Adsorption Assembly

discs are held on the upper faces of the flanges closing off the adsorbent chambers. This flange seats on the rim of the syringe and is held in place by means of a flange nut.

The syringe is a hollow metal cylinder, 16 inches long by 3 inches in diameter, threaded at both ends. The upper end is closed by a flange which holds the nipple for the air connector and is held on similarly to the bottom flange.

A light, close fitting piston rides within the cylinder.

The faces of all these parts are countersunk to take gaskets to make all the seals tight.

Stainless steel is used throughout. The whole assembly is screwed into the top of the prism and supported by a clamp (cf. Figure IV).

Analytical Adsorption Assembly - Figure IV and Figure VII

All the filters for this assembly are made to fit directly into the top of the prism. These are of similar construction to the above. Four filters were constructed; their sizes being; No. 1, 3/4 inch long by 3/16 inch in diameter; No. 2, 1 inch long by 3/8 inch in diameter; No. 3, 2 inches long by 3/8 inch in diameter; and No. 4, 4 inches long by 3/8 inch in diameter.

The No. 1 filter has a 1/4 inch long by 3/32 inch channel from the adsorbent chamber to the prism inlet channel. The perforated disc which rests on the bottom of the adsorbent chamber adds another 1/8 inch space which can be used together with the 1/4 inch space as a mixer chamber when filled with pyrex mesh. The upper end of the filter has a large flange

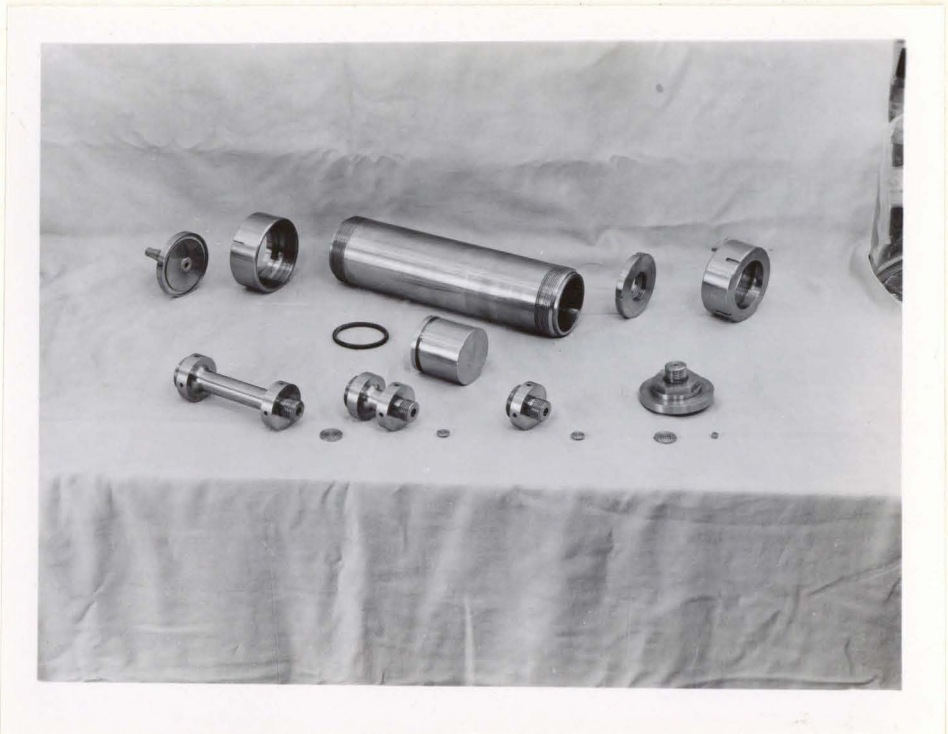


Figure VII

Filters and Syringe Apparatus for the
Analytical Adsorption Assembly

which fits the syringe and is held on by a flange nut. The top perforated disc sits countersunk in the flange and closes off the adsorbent chamber. The remaining three filters have a perforated disc, 1/4 inch in diameter, which rests on a shoulder at the bottom of the adsorbent chamber. The tops of these three filters are threaded and screw into a flange. A perforated disc sits countersunk on top of the filter. The flange rests on the syringe and is held in place by a flange nut.

The syringe is a hollow metal cylinder, 8-1/4 inches long by 1-3/4 inches in diameter. The upper end is closed by a flange which holds a nipple for the air connector and is fastened as is the bottom flange.

The light close fitting piston, which has a groove cut to hold an O ring, rides within the cylinder. Teflon and polythene gaskets are used between all interfaces.

The assembled adsorption apparatus is screwed into the top of the prism and is held in place by means of a clamp (cf. Figure IV).

Air Pressure Regulator

The air is passed through a filter to clean it and then through a pressure regulator provided with a manometer (0-80 p.s.i.) (cf. Figure V). This regulator leads to a three way valve for releasing pressure. A rubber hose leads from the valve and has a connector on the end which fastens onto the nipple on top of the syringe.

Holders for the Syringes

Two holders fastened to a table are provided to aid in filling the syringes and clamping them so pressure can be applied to tighten all joints. A set of wrenches designed to fit the various parts is provided.

Section 2 Operational Mechanics for the Analytical Adsorption Assembly for Frontal Analysis

The desired filter is packed with adsorbent and weighed before and after packing. If the weight of the adsorbent differs too much (e.g., 5%) from the normal value the packing is irregular and a diffuse boundary would be obtained in the experiment. The filter is packed in the following manner. The metal perforated disc is placed in the filter and then a filter paper of the proper size is placed on top of this. Suction from a water aspirator (20-30 mm. pressure) is now applied to the bottom of the filter and the adsorbent is added with occasional tapping with a tamper. The countersunk area is cleaned of excess adsorbent, a filter paper placed on top of the column and then a second perforated disc placed in position. The filter is turned over (e.g., No. 1) and the small exit hole is filled with pyrex glass, mesh 40-60, which acts as a mixer. The end is stoppered with cotton or glass wool.

The filter paper discs must be made to fit exactly or else carbon will be forced into the prism. It was found that Eaton-Dikeman Filter Paper No. 615 cut with a No. 1 and No. 10 cork borer produced tight fitting papers which worked well for filter No. 1. No. 5 and No. 10 cork borers cut papers suited for the other three filters.

The syringe is placed in the upright position in the holder, the O ring on the piston is wet with the solvent and the piston pushed into the syringe to the desired depth.

A rod conveniently marked as to the depth for certain volume units is used. The solvent was freed from air (in a vacuum) for two reasons. First, the dissolved air forms bubbles in the prism channel where there is no extra pressure, thus rendering observation impossible. Second, even if observation were possible the index of refraction of the solution would be a function of the amount of dissolved air and the readings would be erroneous. The O ring was necessitated by the fact that the air channeled around the piston and caused bubble formation in the prism by being forced into solution. The seal of the O ring can be tested; if no air bubbles emerge after all the solution is forced out through the filter the O ring is intact. If the O ring is cracked bubbles can be seen passing through the prism. Preliminary tests on the Preparative Adsorption Assembly showed air to bypass the piston, therefore an O ring should also be placed on this piston.

The syringe is now carefully filled to the rim with solvent. It is tapped to remove any entrapped air bubbles. The filter is carefully mounted so as to avoid any air bubble formation, with a polythene gasket between the flange and the syringe, and the flange nut put in place and tightened. The syringe is dropped to the horizontal position in the holder and the flange nut tightened with a wrench.

The air pressure connection is attached and sufficient solvent forced through the filter to displace all the air,

15 ml. required for filter No. 1. When this is accomplished the air pressure is slowly released to gradually take up the spring caused by the distortion of the O ring. If this is done rapidly a back suction will draw air into the filter. Next the flange nut is removed and the syringe emptied. The syringe and piston are air dried, reassembled wetting the O ring with solution and the syringe filled with the solution to be analysed. The filter is then connected in the above fashion. The excess solution is thoroughly removed from the outer edges and the assembly is dried. This is to prevent contamination of the bath.

The prism-solvent cell assembly is assembled as follows (conveniently done while washing the filter free of air).

The small inner window is placed in position first. Two polythene gaskets are set on the shoulder, and then the small window placed in position. The window is handled by means of tweezers and is washed with solvent after removing it from the water in which it is stored. All windows are cleaned with cleaning solution (sodium dichromate and sulfuric acid), washed, and kept under water. In this manner they were found to remain clean and are easily wet by the solvent. If the windows are dried first bubble formation is facilitated. A metal gasket is placed on top of the window and a small hollow screw is tightened on by hand. Care in placing the window and metal gasket is profitable, for if either is caught in the threaded walls, the window cracks on tightening the screw.

The solvent cell is rinsed with solvent and then filled to the brim by means of a dropper. A polythene gasket is next placed in the recess and a previously solvent washed window is slid into place, avoiding bubble formation. The screw on the cell holder (cf. Figure II) is loosened, the metal frame assembly turned upside down, slanting away from the side of the cell being placed on the frame. A polythene gasket is wet with solvent and placed over the prism channel hole. The solvent cell is slid carefully into position. It is raised slightly off the base, by sliding it on the 45^{o} face, (to prevent catching on the base and forming air bubbles when the screw is tightened) and the screw is tightened on the holder, forcing the cell flush against the prism face. This is repeated with the second solvent cell. The solvent cell to be placed on the side which has the capillary exit tube from the prism, is marked with an O scratched on the outer metal surface.

The assembly is then uprighted. The capillary tube is now tightly placed in its seat by rotating it and applying pressure to the elbow. Then it is clamped in place by a screw apparatus on top of the assembly. A teflon gasket is placed in the well for the filter on top of the prism and then filled with solvent. A teflon gasket was found more suitable than polythene as the latter gasket deformed when pressure was applied and in the larger cells caused the perforated disc to be displaced upwards causing the adsorbent

to fall into the prism. After the well is filled with solvent, the bubbles in the prism are removed by suction. The channel is checked for air bubbles by peering through the solvent cells with the aid of a light. If bubbles are present in the solvent cells the above procedure is repeated. The capillary tube is filled by suction and a dropper bulb placed over the end to prevent the liquid from running out.

The well on top of the prism is filled to the brim with solvent and the syringe plus filter placed loosely in the clamp and screwed into place. The prism channel is watched as this is tightened to see that no bubbles are formed. When the filter is three-quarters screwed in, the dropper bulb is removed from the capillary tube and the filter screwed in tightly, final tightening with a wrench. Then the clamp on the syringe is tightened. The apparatus is now completely dried of solvent (unless water is the solvent) to prevent contamination of the bath. The outer part of the solvent cell is filled completely with water, avoiding bubbles, and the whole assembly is placed in the waterbath. Often there are air bubbles found in the water in the outer channel of the solvent cells and these can be removed by blowing into a bent tube placed in the outer cell. By viewing the cell from the side of the light source, bubbles are easily detected.

It is recommended that the distilled water in the bath be changed frequently (every three or four runs) as it is easily contaminated by the assembly. The effect of variation

of the bath temperature was checked over an hour's period and a change in temperature of 0.06° did not cause any significant change in refractive index. It is to be stressed however that although the bath temperature changes little with room temperature variation, the change in density of the air by these variations can often be detected. Therefore extreme care in keeping the room temperature constant is to be emphasized.

In the work done to date the assembly was allowed to stand one and one-half hours to come to complete thermal equilibrium. It is recommended that the solution to be analysed be previously brought to bath temperature, as often a few degrees difference will cause one ml. of solution to be forced out during equilibration due to expansion. It is very important that thermal equilibrium be established. Drifting of the refractive index while equilibrium is being reached may cause differences larger than those anticipiated between fronts.

The air hose is connected, the proper pressure applied and the valve turned on. The pressure used depends on the size of the cell and adsorbent used. For a Norite packed No. 1 filter, a pressure of 30 p.s.i. gave a rate of 1 ml./ two minutes.

The rate of flow is an important variable as shown by Hellstrom[26]. Too low a rate may cause a small extra front while too great a rate causes an extra increase in refractive index which falls again after the front becomes steady.

During the experiment the slide is continuously moved to keep the eyepiece focused on the cross hair and readings are taken corresponding to certain volume elements. These readings are converted into differences of refractive index and later plotted against volume. Corrections for differences in amounts of adsorbents, volume of the capillary tube, and volume held in the adsorbent are applied[6] to obtain the specific retention volume, which is necessary for calculations and comparisons between different experiments.

The active charcoal used as an adsorbent, Braun-Norite A Lot No. 21296, was purified by washing with distilled water and ethanol according to Claesson[6]. Some charcoal was reactivated by heating at 1000° for seven hours but no improvement in adsorption could be detected.

Mention has been made of the mixer arrangement for filter No. 1 in the Analytical Adsorption Assembly. The necessity for such an adaption was soon encountered, in that for concentrated fronts, i.e., large changes in refractive index, the line became very hazy in the eypiece and could not be followed until the front had passed and a steady concentration was again passing through the prism. The mixer of Hagdahl[9] was tried and though the impinging front could be read continuously, the solution was so mixed that no sharp front could be detected. A half packed mixer was tried with better success. However, still too much mixing was apparent. Consequently the mixer described above for filter

No. 1 was found to be the most satisfactory, in that the front could be continuously followed and the fronts were not smeared out.

The important features readjusted on the instrument as delivered from the shop were:

1. Correction of the cross hair position,

2. Change of magnification of the eyepiece from 10x to 3.5x, which increased the sharpness of the cross hair,

3. The method of holding the solvent cells was altered, which permitted assembly without air bubbles and leaking of the cells.

4. Adaption of proper gaskets. Teflon is not sufficiently compressible to make absolute seals at all interfaces while polythene is. Teflon was retained in one position (p. 181).

5. Placing the O ring on the piston to make an air tight seal.

6. Adaption of mixer.

Section 3 Applications - using the Analytic Adsorption Assembly.

Frontal analysis of fatty acids was first attempted. Successful separations were obtained with a solution of 0.075% myristic acid and 0.150% stearic acid in absolute ethanol using filter No. 1 packed with Norite A (cf. Figure VIII). When unpurified stock room chemicals were used for a mixture of 0.075% palmitic acid and 0.075%

Figure VIII

Frontal Analysis of 0.075 gm. Myristic Acid and 0.150 gm. Stearic Acid/100 ml. Absolute Ethanol

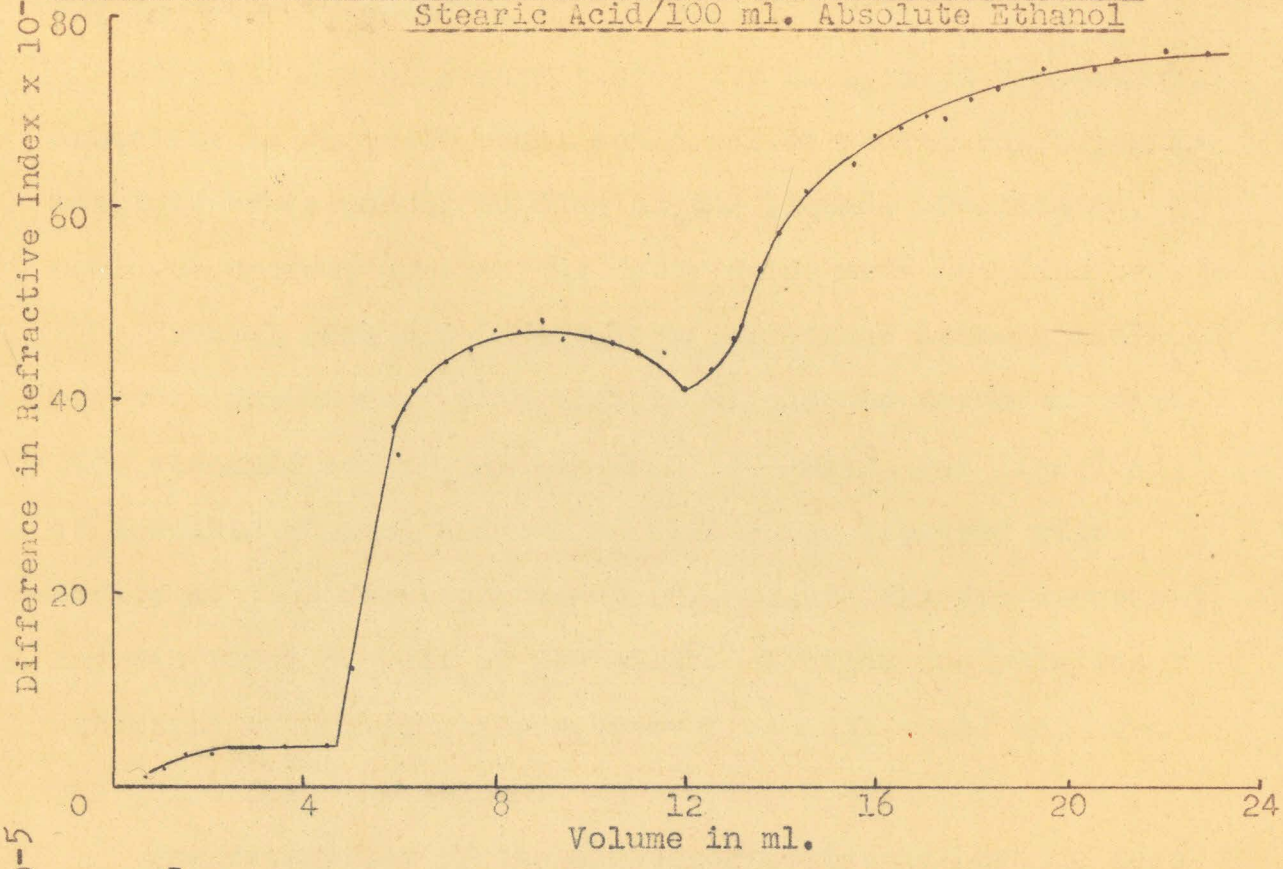
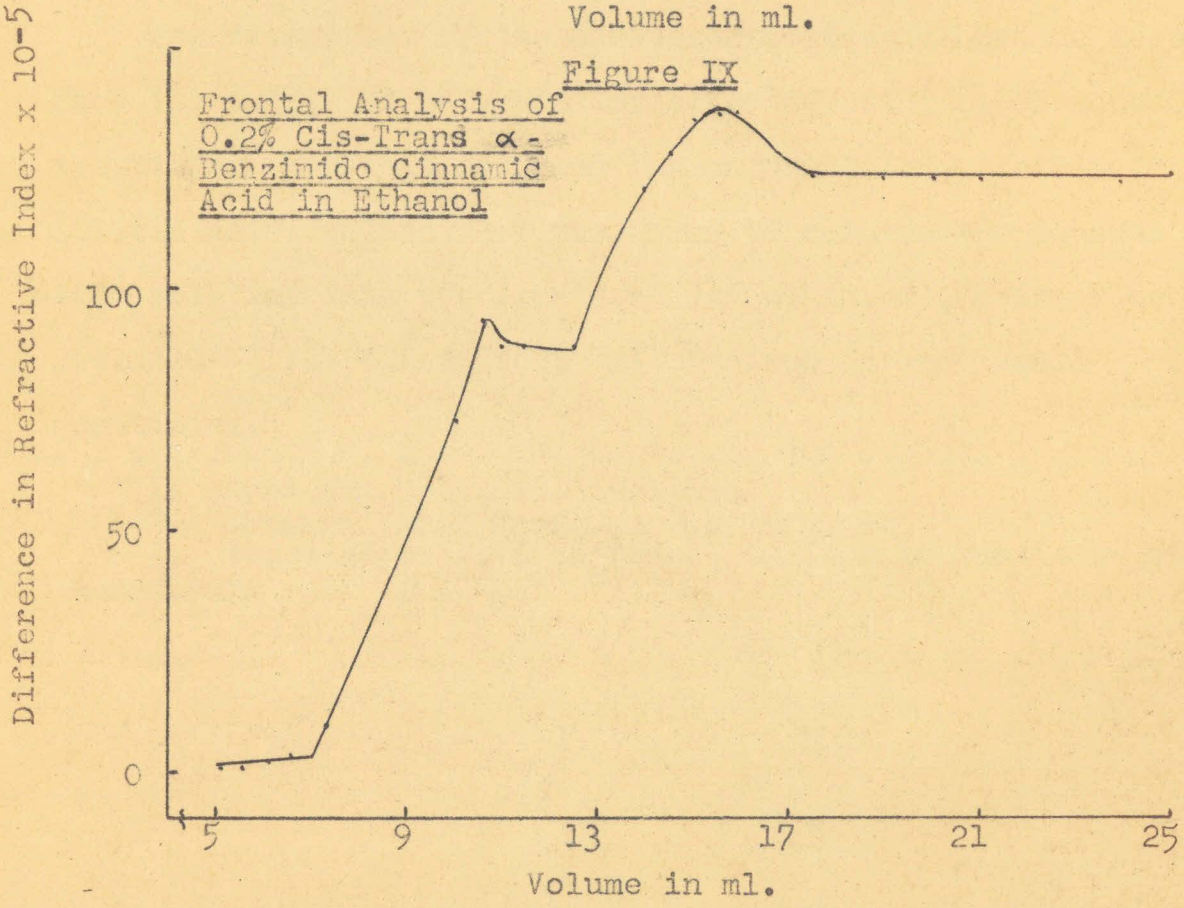


Figure IX

Frontal Analysis of 0.2% Cis-Trans α-Benzimido Cinnamic Acid in Ethanol



stearic acid in ethanol, three fronts were observed, showing an impurity present.

Two basic resins were tested for fatty acid separation. Amberlite IR 4B[1], 120 mesh, could not be packed sufficiently tight to cause enough hold up of the liquid. Duolite No. 2[2] could be packed; however the fatty acids were not adsorbed.

Frontal analysis of a pair of cis-trans isomers isolated one pure component. This mixture of cis and trans α benzimido cinnamic acids[3] melting from 187°-212°, was dissolved in absolute ethanol and run on filter No. 1 packed with Norite A. The first component (cf. Figure IX) was recovered and was found to melt at 212°-213°. Elution analysis was attempted with this mixture, however ethanol would not move the acids down the column.

The resolution of the adrenocorticotropic hormone (see Part II) sample RN 147, was investigated using 0.05% aqueous solutions. Norite A adsorbed the material irreversibly. Silicic acid: celite, 3:1, was found to resolve the aqueous ACTH solution into two fronts as did cellulose, Solka Floc B.W. 200. The first front was obtained in very small quantities.

- -

1. Rohm and Hass, Philidelphia.
2. Obtained from Professor C. Niemann.
3. Supplied by J. L. O'Brien, California Institute of Technology.

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PART IV

THE EFFECT OF DIPHENYL HYDANTOIN (DILANTIN) ON ETHER NARCOSIS IN THE CAT

THE EFFECT OF DIPHENYL HYDANTOIN (DILANTIN) ON ETHER NARCOSIS IN THE CAT

Introduction

The thesis that various parts of the neuron differ in sensitivity to oxygen lack led van Harreveld[1] to postulate and confirm that a "depolarization potential" could be led off during cord asphyxiation. This depolarization potential is due to the fact that certain grey matter elements in the spinal cord, e.g. nerve cells, are depolarized more rapidly by oxygen lack than the axons and other white matter which have much lower oxygen requirements[2,3]. It was found that the grey matter developed a negativity with respect to the anterior root or posterior or lateral column.

Several investigations[4] showed that narcotics effect the polarization state of the peripheral nerve; using the depolarization potential as an indicator, van Harreveld[4] observed the effect of narcotics on the central nervous elements. Depolarization can only be detected if part of the neuron depolarizes. For uniform depolarization of the complete neuron no effect is observed. Therefore to measure the polarization state, the asphyxial depolarization potential was employed.

From the observation that diphenyl hydantoin (dilantin) in a dose of 50 mg. per kg. bodyweight depresses the asphyxial depolarization potential the conclusion was drawn that this

compound has a depolarizing action on the nervous elements in the spinal cord of cats. Since the administration of dilantin does not produce a depolarization potential, it was further suggested that the spinal neuron is depolarized uniformly(5). Asphyxial depolarization differs from the dilantin induced depolarization in that it affects first the neuron parts which are more sensitive to oxygen lack(1). The administration of dilantin has no marked effect on the kneejerk or the flexion reflex of the lightly narcotized preparation.

The narcotic dose producing comparable states of nervous depression caused varying degrees of depression of the asphyxial depolarization potential for the narcotics diallyl barbituric acid, chloral, etc. Cocaine, which causes increased spinal reflex activity, was also found to cause depression of the depolarization potential. These experiments(5) showed that synaptic conduction is not incompatible with a considerable uniform depolarization of the spinal neuron. Earlier work had shown that the depolarization and membrane stabilizing effects of narcotics are not related(6).

Ether has a pronounced depolarizing action on peripheral nerve(7,8,9), as well as on spinal cord elements(4). Ether also depolarizes the spinal neuron uniformly and its effect is therefore similar to the depolarization caused by dilantin. Lorente de No(9) succeeded in restoring conduction in peripheral nerve narcotized with ether, by repolarizing the nerve

membrane with an externally applied anodal current. This indicates that depolarization is an important factor in the narcotic effect of ether on peripheral nerve. Assuming the same to be true for the narcotic effect of ether on central synaptic conduction, it can be expected that diphenyl hydantoin, which itself does not depress spinal reflex action, but which depolarizes cord elements, will potentiate the narcotic effect of ether on reflex activity.

The central narcotic effect of pentobarbital does not seem to depend on depolarization. Pentobarbital in narcotic doses (50 mg. per kg. bodyweight) causes only a moderate depolarization of the spinal cord elements(4). Eccles(10) and Brooks and Eccles(11) found that pentobarbital hampers the elaboration of a conducted impulse from the synaptic potential. Increasing the amount of pentobarbital administered necessitates increasingly higher synaptic potential for the initiation of a conducted impulse in the motoneurone. Finally even the fully developed synaptic potential becomes incapable of eliciting a conducted impulse. Since the narcotic effect of pentobarbital thus seems to depend not on depolarization but on a "membrane stabilizing effect", there is less reason to expect a potentiating effect of dilantin on pentobarbital narcosis.

Van Harreveld(12) found that there was no evidence of a potentiation of pentobarbital narcosis in the cat by diphenyl hydantoin. Not only was in no instance a potentiation of the narcotic effect of pentobarbital by dilantin observed,

but in most experiments there were indications of an antagonism between these drugs, which resulted in an increase of the kneejerk contractions after diphenyl hydantoin administration. This seems to indicate that depolarization is of little importance for the narcotic effect of pentobarbital, since even the combined depolarizing action of this compound and of dilantin are insufficient to depress the kneejerk.

Section 1. Discussion of Results

The work reported in this thesis is an investigation of the effect of dilantin on ether narcosis in the cat.

The effect of the injection of diphenyl hydantoin in a dose of 50 mg. per kg. bodyweight during a series of determinations is shown in Fig. I (Table 7). The four control determinations before the injection showed only minor variations. The first two determinations after the injection were about 40% lower than the control values. From then on the minimum ether concentration necessary to suppress the kneejerk increased again to reach the original level after about an hour. It was found constantly that the greatest depression of the minimum ether concentration was not reached immediately after the injection of dilantin, but 20-40 minutes later. It was surprising that the effect of dilantin on the narcotizing ether concentration is of relatively short duration (not more than 60 to 90 minutes). Table 1 shows the results obtained in 5 experiments of this kind. The mean decrease of the minimum ether concentration to narcotize the

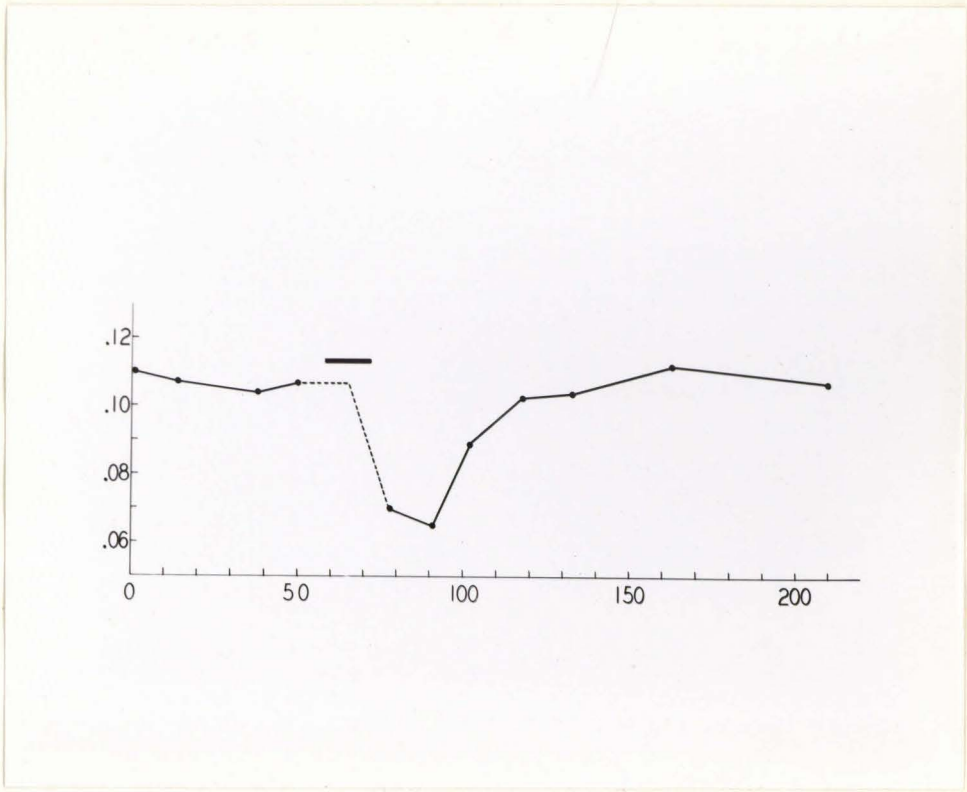

Figure I

The Effect of Dilantin on Ether Narcosis of the Knee Jerk of the Cat

Ordinate - percentage ether in blood.
Abscissa - time in minutes.
Bar, 58-72, represents the time of injection of dilantin.

kneejerk due to the administration of dilantin was 44 ± 2%.

Preliminary experiments summarized in Tables 2-4 had shown that there was an induction period for the ether potentiating effect of dilantin. It was also noted that a concentration of greater than 25 mg. per kg. bodyweight was necessary for the ether potentiating effect of dilantin. Complete recovery from the effect of dilantin upon ether narcosis in a relatively short time was observed. An early observation indicated that for intravenous injection of dilantin, water was a better diluent than Ringer's solution in that circulatory collapse was less frequent.

Tables 5-10 contain data for experiments wherein dilantin was administered both intra-arterially and intra-venously in a concentration of 50 mg. per kg. bodyweight.

In two control experiments (Tables 11 and 12) the amount of alkali which would be necessary to dissolve diphenyl hydantoin in a dose of 50 mg. per kg. bodyweight was administered. In these experiments the minimum ether concentration required to narcotize the kneejerk had a tendency to slowly and gradually increase in time. These experiments demonstrate the expected potentiating effect of diphenyl hydantoin in a dose of 50 mg. per kg. bodyweight on the narcotic effect of ether on the cat although given alone dilantin does not depress spinal reflex activity.

There is another important effect of the injection of dilantin. This is an immediate increase in the size of the kneejerk. This phenomenon is more clearly demonstrated in

those experiments in which the drug was injected swiftly into the peripheral circulation. Figures II and III demonstrate that the increase in strength of the kneejerk is not an artifact. From the first triangle to the second on Figure II dilantin was injected into the manometer circuit. At the third this solution was expelled into the peripheral circulation and at the fourth the ether apparatus was turned on. The time interval between the third and fourth was approximately one minute. Figure III, upper trace depicts the effect of merely shutting off the supply of ether while the lower trace is for a control experiment where 10 ml. of 0.2% sodium hydroxide was injected intra-arterially. The triangles mark the time the ether was turned off and sodium hydroxide injected respectively.

From their effect on the asphyxial depolarization potential it was concluded that both diphenyl hydantoin and ether have a depolarizing action on the nervous elements in the spinal cord[4,5]. Although diphenyl hydantoin given alone has little effect on spinal reflex activity it does potentiate the narcotic effect of ether as has been shown above. This supports the thesis that the depolarizing effects of ether and dilantin are additive and that depolarization is an important factor in the narcotic effect of ether on central conduction. Although depolarization seems to play an important part in the effect of ether on central as well as on peripheral[9] nervous structures, it is not the only effect of this compound. The threshold of excitation of

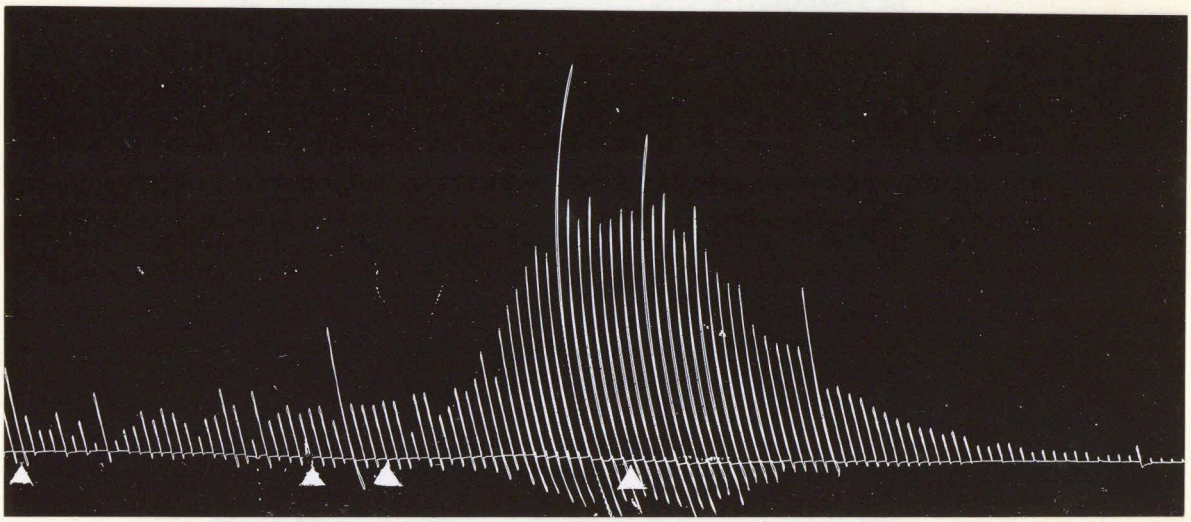

Figure II

To Illustrate the Initial Increase of the Knee Jerk Upon Dilantin Injection.

First to second triangle - dilantin injection into the manometer: third triangle - solution expelled into the peripheral circulation: fourth triangle - ether administered.

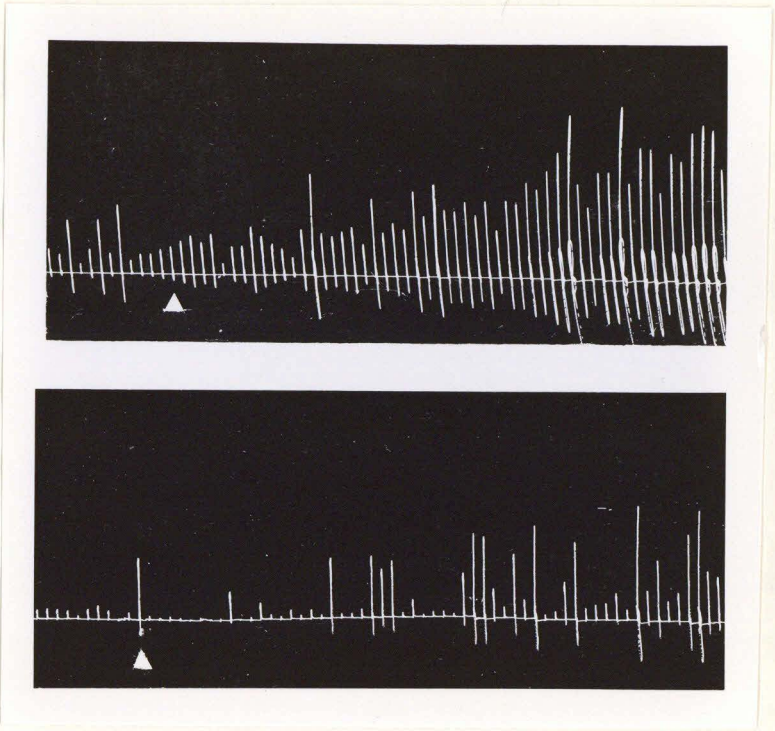

Figure III

Control Experiments with Ether and Sodium Hydroxide

Upper trace: Ether control. At triangle ether was turned off.

Lower trace: Sodium hydroxide control. At triangle the ether was turned off and sodium hydroxide added.

peripheral nerve increases vary considerably when subjected to ether vapors[7]. This is not due to the resulting depolarization since asphyxial depolarization of peripheral nerve was found to produce either a decrease of the threshold[13,14] or to leave the excitability unchanged[7]. It seems therefore that ether has a "membrane stabilizing" effect in addition to its depolarizing effect. Both effects may cooperate in producing the narcotic effect of ether.

The effect of diphenyl hydantoin on ether narcosis has been shown to be quite different from its effect on pentobarbital narcosis.

Section 2 Experimental and Data

The animals were prepared under light ether narcosis. The neck was opened and the trachea immediately canulated so that artificial respiration could be administered when necessary. The left external jugular was canulated to facilitate the administration of the drugs and Ringer's solution required to maintain the blood pressure. The left carotid was cleaned so that samples of blood could be removed for ether determination. The right carotid was then canulated in order that the blood pressure could be read continuously. The animal was made spinal by ligating the dura at Th10-Th12. The condyle of the right femur was drilled to take a 3/16" screw. The femur was then fastened to a wooden brace by means of a screw, taking care not to impair the movements of

the shank. The kneejerk was elicited by an electro-magnetic hammer(15) which tapped the quadriceps tendon at regular intervals (ca. 3 sec.). The blood pressure manometer was connected and the movements of the shank were recorded on a kymograph.

Ether was administered by passing the air for artificial respiration over a bottle of ether resting in a warm water bath. The bottle had a controllable by-pass for fine regulation of ether concentration. The minimum ether concentration in the blood necessary to suppress the kneejerk was determined before and after the administration of dilantin. An ether concentration was offered in the respired air sufficient to decrease slowly and finally to suppress the kneejerk (Figure IV - 1A, 1B, 1C). At the moment that the tap on the tendon failed to elicit a reflex contraction a 2 cc. sample of blood was taken from the carotid artery. A 2 cc. pipette was connected by a small length of rubber hose with a #19 hypodermic needle. The needle was forced through the arterial wall and the arterial pressure filled the pipette quickly and with a minimum loss of ether from the blood. The ether bottle was removed from the respiratory path by a clamp at the beginning of the sampling. The animal was allowed to recover from the deep narcosis until the kneejerk returned to a moderate size. After this the ether bottle was again placed in the path and another sample taken at the proper time. In this way a determination of the minimum ether concentration

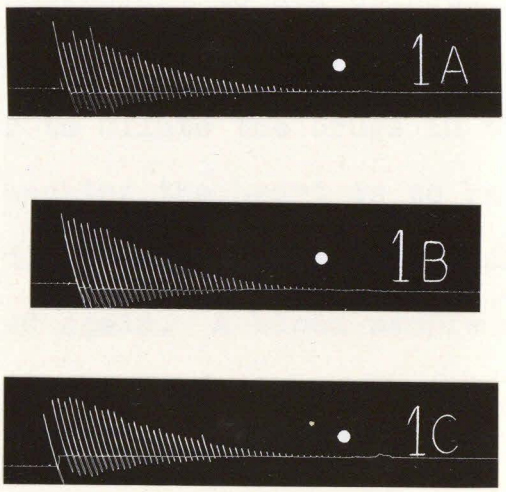

Figure IV

Effect of Ether on the Knee Jerk

At the circles, where the knee jerk failed, blood samples were taken.

in the blood necessary to narcotize the kneejerk was made at 10 to 15 minute intervals. The animal was then allowed to come under a fairly light narcosis and dilantin was added through either the jugular vein or the right carotid. The usual dose was 50 mg. per kg. bodyweight dissolved in a 0.2% sodium hydroxide solution. When added intra-venously a time interval of 10-15 minutes was necessary as the blood pressure dropped too fast if there was a shorter interval. When added arterially, the solution was added to the clamped manometer tube and then forced into the peripheral system suddenly. It was necessary to add epinephrine (10^{-5}) through the jugular to combat a pressure decrease. Intra-arterial injection in order to dilute the drugs in the blood of circulation before reaching the heart is to be preferred. After injection of the dilantin the ether was administered until the kneejerk failed again. A blood sample was withdrawn as before for analysis. This was repeated in fifteen minute intervals as many as eight times. In order that the rate of narcosis would be approximately the same each time, the control valve was not touched when the animal was recovering. Rather, a clamp was applied to the tube going to the ether bottle. Cats were used exclusively in these experiments.

Ether determinations were made essentially in the manner of Friedman[16]. By this method the ether in the samples was determined to within $\pm$ 1%.

Table I

Minimum Concentration of Ether (gm. Ether/gm. Blood x 100) in the Circulating Blood, Necessary to Suppress the Kneejerk

No.	Mean of the Control Values	Lowest Value after Diphenyl Hydantoin Administration[1]	% Decrease of Ether Concentration	Time of Minimum (minutes)
1	0.088	0.052	-41%	30
2	0.132	0.065	-51%	50
3	0.107	0.065	-39%	25
4	0.052	0.028	-46%	30
5	0.122	0.070	-43%	20
		Mean	-44%	

1. 50 mg./kg. bodyweight

Note: Time taken for the minimum is taken from the time at the middle of the injection to the lowest value obtained for the ether required for the abolishment of the knee reflex.

Tables 2 - 10

Ether Concentration vs Time for Dilantin Injection

Unless otherwise stated the Dilantin concentration was such that 10 ml. contained 50 mg./kg. bodyweight in 0.2% NaOH and was added intravenously

Table 2

Sample No.[1]	% Ether
1	0.160
2	0.152
Dilantin added[2]	
3	0.129
Dilantin added[2]	
4	0.092
5	0.086

Table 3

Sample No.[1]	% Ether
1	0.129
2	0.140
3	0.127
Dilantin added[3]	
4	0.139
5	0.129
Dilantin added[3]	
6	0.141
Dilantin added[3]	
7	0.114
Dilantin added[3]	
8	0.121
Dilantin added[3]	
9	0.127
Dilantin added[3]	
10	0.115

1. Fifteen minutes between consecutive ether samples. Dilantin given over fifteen minute intervals. Ether samples taken approximately five minutes after dilantin injection
2. 35 mg./kg. bodyweight - dissolved in Ringer's solution plus sufficient NaOH to dissolve the sample.
3. 12.5 mg./kg. bodyweight - dissolved in H_2O plus sufficient NaOH to dissolve the sample.

Table 4

Sample No.[1]		% Ether
1		0.100
2		0.098
3		0.113
4		0.098
	Dilantin added[2]	
5		0.085
6		0.093
7		0.100
8		0.100
	Dilantin added[3]	
9		0.058
10		0.082
11		0.096

Table 5

Time Sample Taken (minutes)		% Ether
0		0.091
15		0.089
31		0.086
48		0.086
63-78	Dilantin added	
84		0.058
100		0.052
111		0.070
127		0.075
143		0.084
161		0.084

1. See footnote 1 on previous page.
2. 50 mg./kg. bodyweight - dissolved in 0.1% NaOH.
3. 25 mg./kg. bodyweight - dissolved in 0.1% NaOH.

Table 6

Time Sample Taken (minutes)		% Ether
0		0.133
18		0.133
37		0.124
55		0.137
106-115	Dilantin added	
129		0.091
144		0.098
160		0.065
177		0.070
198		0.079
212		0.091

Table 7

Time Sample Taken (minutes)		% Ether
0		0.110
16		0.107
38		0.104
50		0.107
58-72	Dilantin added	
78		0.070
91		0.065
102		0.089
118		0.103
133		0.103
187		0.112
212		0.107

Table 8

Time Sample Taken (minutes)		% Ether
0		0.079
30		0.052
41		0.084
57		0.093
65-80	Dilantin added	
86		0.084
98		0.084
112		0.075
125		0.070
138		0.058
151		0.051
166		0.068

Table 9

Time Sample Taken (minutes)		% Ether
0		0.061
14		0.047
26		0.049
36		0.051
44-45	Dilantin added[1]	
50		0.047
62		0.037
73		0.028
84		0.033
95		0.033
107		0.037
122		0.028
133		0.040

1. Intra-arterially.

Table 10

Time Sample Taken (minutes)		% Ether
0		0.149
12		0.110
26		0.121
38		0.107
56-57	Dilantin added[1]	
62		0.086
73		0.070
85		0.098
98		0.107
110		0.098
125		0.112

Table 11

Ether Concentration vs Time for NaOH Blank (0.2%) 10 ml.

Time Sample Taken (minutes)		% Ether
0		0.135
24		0.126
41		0.154
53		0.158
64		0.145
84		0.171
90-99	NaOH added	
108		0.175
124		0.161
137		0.182
150		0.191

1. Intra-arterially

Table 12

Ether Concentration vs Time for NaOH
Blank 0.2%-10 ml.

Time Sample Taken (minutes)		% Ether
0		0.140
15		0.117
22		0.112
32		0.168
45-55	Ether Recovery	
60-70	NaOH added	
79		0.117
90		0.135
98		0.149
111		0.135

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PROPOSITIONS

1. The view that peptic and chymotrypsin hydrolysis follows an "all or none" type of degradation rather than a gradual breakdown has been expressed[1,2]. It is proposed that the validity of this hypothesis can be challenged on the basis of other studies[3] and that the phenomenon observed is one concerned with relative rates of hydrolysis.

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2. It is proposed that the mode of activation and the activated complex of chymotrypsin hydrolysis of esters and amides could be further investigated by the use of heavy water[1-5]. Knowledge of inhibition mechanisms by D isomers could also be obtained. Experiments to this end will be proposed.

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(5) Bender, M. L., J. A. C. S., 73, 1626 (1951).

3. It is proposed that the observed inhibition by products[1,2] of the urease catalysed hydrolysis of urea can be redefined as being an extension of the type of non-specific inhibition due to the alkali metals[3]. It is further proposed that the alkali metals, rubidium and cesium will also inhibit urease action.

(1) Laidler, K. J., Hoare, J. P., J. A. C. S., 71, 2699 (1949).
(2) Hoare, J. P., Laidler, K. J., J. A. C. S., 72, 2487 (1950).
(3) Fasman, G. D., Ph. D. Thesis, California Institute of Technology, 1952.

4. The theory that urea combines with a substance X to form a compound which could be hydrolysed by urease was advanced by Kato[1]. It is proposed that recent evidence[2-4] adds weight to the validity of this hypothesis. Inhibition

by high concentrations of substrate[5] can also be easily explained by this theory.

(1) Kato, N., Biochem. Z., 136, 498 (1923).
(2) Fasman, G. D., Ph.D. Thesis, California Institute of Technology, 1952.
(3) Pauling, L., "Nature of the Chemical Bond", p. 212, Cornell University Press, Ithaca, N.Y., 1945.
(4) Schuck, A. F., Ph.D. Thesis, California Institute of Technology, 1950.
(5) Laidler, K. J., Hoare, J. P., J. A. C. S., 71, 2699 (1949).

5. An experiment will be proposed to test which of two theories for the active complex of the urease catalysed hydrolysis of urea is valid. The Laidler theory[1] proposes a urea-urease-H_2O complex, while the Fasman[2] theory proposes a urease-urea-activator complex.

(1) Laidler, K. J., Hoare, J. P., J. A. C. S., 72, 2489 (1950).
(2) Fasman, G. D., Ph.D. Thesis, California Institute of Technology, 1952.

6. The complex ionization of a number of carboxylic acids, e.g. 2,4,6 trimethylbenzoic acid[1,2], in sulfuric acid has been demonstrated by cryoscopic measurement. It is proposed that the carbonium ion formed in this manner could also be detected by a coupling reaction similar to the Friedel-Crafts reaction.

(1) Treffers, H. P., Hammett, L. P., J. A. C. S., 59, 1708 (1937).
(2) Newman, M. S., Kuivila, H. G., Garrett, A. B., J. A. C. S., 67, 704 (1945).

7. The difficulty of detection of small quantities of proteins has hindered the advance of paper chromatography of protein mixtures. It is proposed that the Pauly test[1,2] would be widely applicable for this purpose, as this test does not depend on a free amino group but rather the formation of a hydroxyazo compound is responsible for the positive color test[3].

(1) Macpherson, H.J., Biochem. J., 36, 59 (1942).
(2) Pauly, H., Z. physiol. Chem., 45, 159 (1905).
(3) Fasman, G. D., Ph.D. Thesis, California Institute of Technology, 1952.

8. The migratory aptitude of alkyl radicals in the 1,2 shift of the Whitmore mechanism for the pinacol rearrangement is dependent on the stability of the carbonium ion formed[1,2]. It is proposed that the relative stabilities of the carbonium ions with various alkyl substituents can be estimated and the final product predicted by invoking the principle of hyperconjugation.

(1) Wheland, G. W., "Advanced Organic Chemistry", p. 504, John Wiley & Sons, Inc., N.Y., 1949.
(2) Day, A. L., "Electronic Mechanisms of Organic Reactions", p. 73, American Book CO., N.Y., 1950.

9. In discussing nucleophilic and electrophilic displacements in the Displacement reaction, Hammett[1] states that "this classification does not permit the qualitative prediction of the effect of the structure on reactivity". It is proposed that such a correlation is possible using the fact that these displacements are S_{N1} and S_{E2} respectively.

(1) Hammett, L. P., "Physical Organic Chemistry", p. 152, Table II p. 154, McGraw-Hill Book Co. Inc., N.Y. (1940).

10. A mechanism will be proposed to account for the formation of p-phenylazobenzene[1,2] from the reaction of benzene and azoxybenzene in the presence of aluminum chloride.

(1) Pummerer, R., Binapfl, J., Ber., 54, 2768 (1921).
(2) Pummerer, R., Binapfl, J., Bittner, K., Schuegraf, K., Ber., 55, 3095 (1922).

11. The work of Glick[1] has shown that urease activity in the gastric mucosa parallels HCl production. It is proposed that the mode of activation of urease[2] is through the phosphate ion present in the parietal cells[3]. Experiments to determine the PO_4 concentration in the mucosa during rest and HCl secretion could test this hypothesis. This mechanism could also serve as a means to keep the charge neutrality in the parietal cell.

(1) Glick, D., Zak, E., Arch. Biochem., 28, 305 (1950); Glick, D., Zak, E., von Korff, R., Am. J., Physiol., 163, 386 (1950); Glick, D., von Korff, R., Am. J. Physiol., 165, 688 (1951); Glick, D., von Korff, R., Ferguson, D. J., Am. J. Physiol., 165, 695 (1951).

(2) Fasman, G. D., Ph.D. Thesis, California Institute of Technology, 1952.

(3) Collip, J. B., University of Toronto, Physiol. Series, No. 35 (1920).

12. Davies'[1] theory of the role of carbonic anhydrase in the gastric mucosa encompasses many of the known facts concerning HCl production in the parietal cells. It is proposed that the work of Apperly[2], Clark[3], and Brown[4] can be cited as confirmatory evidence of this hypothesis while the work of Gray[5] can be seriously questioned.

(1) Davies, R. E., Biochem. J., 42, 609 (1948).
(2) Apperly, F. L., Crabtree, M. G., J. Physiol., 73, 331 (1931).
(3) Clark, B. B., Adams, W. L., Gastroenterology, 7, 284 (1947).
(4) Brown, J. S. L., Vineberg, A. M., J. Physiol., 75, 345 (1932).
(5) Gray, J. S., Adkinson, J. L., Am. J. Physiol., 134, 27 (1941).