traveled as a single spot[14] at R_{ad} 1.36. A sample was air-dried for analysis.

Anal. Calcd. for $C_{11}H_{15}N_5O_4 \cdot H_2O$: C, 44.1; H, 5.73. Found: C, 44.1; H, 6.06.

The base was converted to its hydrochloride, m.p. 173–175°, which gave an infrared spectrum identical with that of the hydrochloride in preparation A.

The filtrate and washings obtained after removal of the 9.33 g. of nucleoside base XXI were combined and taken to dryness *in vacuo*. The residue was dissolved in 250 ml. of methanol and treated with 600 ml. of a 10% solution of picric acid in methanol. The mixture was cooled to 0° for several hours, then filtered. The precipitate was recrystallized from 400 ml. of water to yield 10.1 g. of picrate.

To 300 ml. of water at room temperature was added portionwise the above picrate (10.1 g.) and 60 g. of Dowex 2 (Cl), using magnetic stirring. The filtered solution was treated with Norit, filtered, and taken to dryness *in vacuo*, leaving 3.16 g. of a white solid; λ_{max}^{KBr} 2.95, 3.10–3.20 μ (OH, NH); 5.92 μ (C=NH$^+$); 6.05, 6.18, 6.42, 6.63 μ (C=C, C=N); 8.95, 9.22 μ (C–O–H, C–O–C).

In a paper chromatogram,[14] this product gave two spots of approximately equal intensity (R_{ad} 0.97 and 1.29). The amount of nucleoside in this mixture would correspond to approximately a 7% yield, thus raising the over-all yield to 49% based on 1-acetate XVIII.

2,6-Diacetamido-9-(2′,3′,5′-tri-O-benzoyl-6′-deoxy-β-D-allofuranosyl)-purine (XXII).—Crude 2,3,5-tri-O-benzoyl-6-deoxy-D-allofuranosyl chloride (XIX), prepared from 2.0 g. (3.86 mmoles) of 1-O-acetyl-2,3,5-tri-O-benzoyl-6-deoxy-allofuranose (XVIII), was dissolved in 50 ml. of dry xylene and added to a suspension of 1.81 g. (3.86 mmoles) of chloromercuri-2,6-diacetamidopurine[13b] mixed with 2.46 g. of Celite[19] in 130 ml. of xylene which had been previously dried by the azeotropic distillation of 50 ml. of xylene. The resulting reaction mixture was refluxed, with stirring, for 3 hours. The hot reaction mixture was filtered and the precipitate was washed with three 15-ml. portions of chloroform. The combined filtrate and washings were taken to dryness *in vacuo* and the residue was dissolved in 25 ml. of chloroform. The chloroform solution was washed with 20 ml. of 30% aqueous potassium iodide solution, then 10 ml. of water. The aqueous phases were back-extracted with 5 ml. of chloroform. The chloroform layers were combined, dried over magnesium sulfate, and taken to dryness to yield 3.0 g. of a yellow residue.

2,6-Diamino-9-(6′-deoxy-β-D-allofuranosyl)-purine (XXIII) Hydrochloride.—To a solution of 3.0 g. of crude 2,6-diacetamido-9-(2′,3′,5′-tri-O-benzoyl-6′-deoxy-β-D-allofuranosyl)-purine (XXII) in 60 ml. of absolute methanol was added 5.44 ml. of 1 *N* methanolic sodium methoxide. The resulting solution was refluxed for 2.5 hours. On cooling, the reaction was neutralized with glacial acetic acid, then taken to dryness *in vacuo*. The residue was partitioned between 40 ml. each of water and chloroform. The aqueous phase was taken to dryness *in vacuo*. The last traces of water were removed by the addition and removal of two 5-ml. portions of absolute ethanol. The residue (1.78 g.) was a yellow-brown solid. The product traveled as a single spot[14] (R_{ad} 0.77) as compared to 2,6-diaminopurine with R_{ad} 0.63.

The crude nucleoside was dissolved in 40 ml. of hot water, and 25 ml. of 10% methanolic picric acid was added. The mixture was cooled to 0° for several hours, then filtered. The crude picrate was recrystallized from 50 ml. of water; *yield 0.80 g., m.p. 189–195° dec.*

The picrate was decomposed in the usual manner in 60 ml. of water with 4.0 g. of Dowex 2 (Cl). The aqueous solution was taken to dryness *in vacuo*, leaving 0.5 g. of cream-colored solid (44% based on 1-acetate XVIII); λ_{max}^{KBr} 2.92, 3.15 μ (OH, NH); 5.87 μ (C=NH$^+$); 6.07, 6.36 μ (C=N, C=C); 7.22 μ (CH_3). The paper chromatogram was identical with that obtained from the crude product. Recrystallization from 23 ml. of 85% ethanol yielded 0.20 g. of white powder, m.p. 210–212° dec., $[\alpha]^{24.2}D$ −76.1° (1.71% in water).

Anal. Calcd. for $C_{11}H_{16}N_6O_4 \cdot HCl$: C, 39.8; H, 5.13. Found: C, 40.0; H, 5.34.

Acknowledgment.—The authors wish to thank Dr. Peter Lim for infrared interpretations and discussions.

MENLO PARK, CALIFORNIA

[CONTRIBUTION FROM THE PALO ALTO MEDICAL RESEARCH FOUNDATION]

Kinetics of β-Glucosidase on the Basis of Intermediate Enzyme–glucoside Formation[1]

BY B. H. J. HOFSTEE

RECEIVED DECEMBER 2, 1957

An analysis is made of the initial rates of hydrolysis of *o*-carboxyphenyl β-glucoside by β-glucosidase at varying substrate concentrations (S) and *p*H. It is found that the *p*H optimum decreases with decreasing S and simultaneously becomes less and less pronounced until for $S \rightarrow 0$ a maximum instead of an optimum occurs. This indicates that the free enzyme carries a single essential ionizable group. The *p*H-optimum can be accounted for by assuming that only the proton-bound enzyme enters into intermediary glucoside formation and that after loss of the proton hydrolysis takes place. The kinetic constants corresponding to this mechanism have been determined by graphical procedures.

In recent years evidence has accumulated that certain hydrolytic enzymes enter into covalent bond formation with the substrate. Some of the experimental observations leading to this conclusion recently have been summarized.[2] If this phenomenon were a general one,[3] it could be postulated on the basis of modern concepts of specificity[4] that in the case of glucosidases an intermediate enzyme-glucoside is formed. Although, to our knowledge, in this case no direct evidence for such a mechanism has been presented, the present analysis shows that the kinetics of β-glucosidase from almonds with *o*-carboxyphenyl β-glucoside as the substrate, are consistent with this assumption.

The data that are analyzed were presented previously[5] to demonstrate the suitability of a direct and continuous spectrophotometric method for the determination of initial reaction rates (v) at varying substrate concentrations (S) and *p*H. It is based on the fact that the light absorption of the substrate at wave lengths around 300 mμ is small compared to that of the reaction product salicylic acid.

(1) Presented, in part, at the 132nd Meeting of the American Chemical Society in New York City, N. Y., September, 1957, under the title "Mutual effect of the substrate and hydrogen ions on the kinetic constants of β-glucosidase." Supported by Grant number C-2289(C3) from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

(2) L. W. Cunningham, *Science*, **125**, 1145 (1957).

(3) See F. Lipmann in "The Mechanism of Enzyme Action" (W. D. McElroy and B. Glass, Editors), The John Hopkins Press, Baltimore, Md., 1954, p. 463.

(4) D. E. Koshland, Jr., *Trans. N. Y. Acad. Sci. Ser. II*, **16**, 110 (1954).

(5) B. H. J. Hofstee, *Arch. Biochem. and Biophys.*, **59**, 398 (1955).

Plotted as v *versus* v/S at a fixed pH (Fig. 1A) these data yield straight lines, one intercept of which equals V_m (v, when S $\rightarrow \infty$), while the slope is K_M (S, when $v = V_m/2$). However, in this manner only a rough estimation of these constants can be made because the data represent a rather limited range of substrate concentrations especially at the higher pH values.[6] For reasons explained previously,[5] the highest substrate concentration used was of the order of 0.01 M. Therefore, at the higher pH values, where K_M is high, the curves are less complete than at lower pH and only the intercept with the abscissa (V_m/K_M) can be estimated with fair accuracy from the graph. The other intercept (V_m) was calculated from

$$V_m = (vV_m/K_M)/(V_m/K_M - v/S)$$

This equation is derived from the Michaelis equation $V_m = v + K_M \times v/S$, on which the plots are based; v and v/S were thereby taken as the average values of those of the experimental points.[7]

The rates at different pH and the same substrate concentration are given by the intercepts of the curves with a line through the origin. Plotted as v *versus* pH it is seen[5] that the pH optimum decreases with decreasing substrate concentration. Simultaneously, however, the pH optimum becomes less and less pronounced until for $S \rightarrow 0$ only a maximum instead of an optimum occurs. This is demonstrated by the fact that V_m/K_M, the first-order rate constant, varies with the hydrogen ion concentration in a simple Michaelis manner (Fig. 1B).

It has been postulated that the occurrence of a pH optimum with respect to V_m indicates that the enzyme substrate complex carries two ionizable groups that influence the activity. This theory has been found to apply to the case of fumarase.[8] The fact that in the present case V_m/K_M does not show a pH optimum suggests that in the free β-glucosidase only one such group is present. The data indicate that if a second group were present its ionization is not influenced by the hydrogen ion concentration up to a pH < 3 where the enzyme is irreversibly inactivated.

Since V_m displays a pH optimum, it could be postulated then that the second ionizable group is the result of combination of the enzyme with the substrate that itself carries an ionizable group. However, with other substrates that do not carry an ionizable group a pH optimum does occur.[9] Furthermore, it is not even necessary to invoke a second ionizable group when an intermediate enzyme-glucoside is assumed to occur. The influence of pH on V_m and K_M can be interpreted on the basis of the postulate that enzyme-glucoside formation occurs only after the enzyme has accepted a proton and that hydrolysis follows upon release of the proton. This postulate is based on the fact that V_m/K_M increases with the hydrogen ion concentration (Fig. 1) indicating that it is the proton-bound enzyme that is the initial active species. The complete process of glucoside formation and hydrolysis could occur only at pH values at which a particular enzyme molecule is part of the time in the ionized and part of the time in the un-ionized form, that is, at pH values of the order of the pK of the ionizable group. On the acid side of this region the enzyme-glucoside would accumulate, while on the alkaline side its formation would be prevented. The occurrence of a pH optimum would be the result.

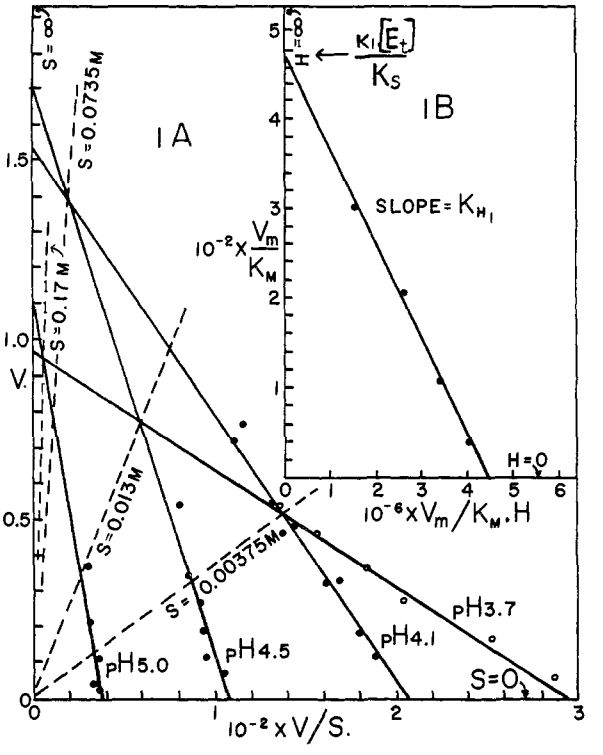

Fig. 1.—Part 1A. Initial reaction rates (v) of the hydrolysis of *o*-carboxyphenyl β-glucoside by β-glucosidase at different substrate concentrations (S) and pH. The intercept of a v *versus* v/S plot with the ordinate ($S \rightarrow \infty$) represents V_m; the slope is K_M. The intercept with the abscissa ($S \rightarrow 0$) equals V_m/K_M. The solid lines are the theoretical ones calculated on the basis of the proposed mechanism and the pertaining kinetic constants. Part 1B. "Michaelis" relationship between the hydrogen ion concentration [H] and V_m/K_M. The slope of the plot gives the hydrogen ion dissociation constant (K_{H_1}) of the free enzyme. The intercept with the ordinate ($k[E_t]/K_S$) is the constant of enzyme-glucoside formation (see text).

The above mechanism implies that the substrate and the proton (H) combine with different sites in the active center. If it is assumed that a primary Michaelis enzyme-substrate complex is formed (see below) whereby S and H do not influence each other's binding[10] one would have

(6) B. H. J. Hofstee, *Enzymol.*, **17**, 273 (1956).

(7) G. S. Eadie, *J. Biol. Chem.*, **146**, 85 (1942).

(8) See R. A. Alberty in "Advances in Enzymology," Vol. XVII (F. F. Nord, Editor), Interscience Publishers, Inc., New York, N. Y., 1956, pp. 49–55.

(9) S. Veibel in "The Enzymes," Vol. I, part 1 (J. B. Sumner and K. Myrbäck, Editors), Academic Press, Inc., New York, N. Y., 1950, p. 593.

(10) Support for this supposition might be drawn from the observations that in the pH range (3.7–5.0) that was applied not only the essential group in the enzyme ($pK_H \approx 4$, see below) but also the substrate ($pK_B \approx 3.6$[5]) occurs partly in the ionized and partly in the un-ionized form. If this ionization were of any consequence in the binding of the substrate, one would expect that K_S and thus K_M (see below) would change with the substrate concentration because the affinities of the two substrate species for the enzyme would be different.

$$E + S \rightleftarrows ES;\ K_S = [E][S]/[ES]$$
$$ES + H \rightleftarrows HES;\ K_{H1} = [ES][H]/[HES]$$
$$HES \xrightarrow{k_1} HE{-}P_2 + P_1$$
$$HE{-}P_2 \rightleftarrows E{-}P_2 + H;\ K_{H2} = [E{-}P_2][H]/[HE{-}P_2]$$
$$E{-}P_2 \xrightarrow{k_2} E + P_2$$

where E is the free enzyme, ES and HES the two forms of the enzyme substrate complex and HE-P_2 and E-P_2 those of the intermediate enzyme-glucoside; P_1 is the product (salicylic acid), the rate of formation of which is measured, and P_2 is glucose. Brackets denote concentrations. The reverse of the reactions represented by k_1 and k_2 are neglected because initial rates were determined and no decrease in rate was observed during the time of measurement.

The active complex HES could equally well be formed *via* the proton-bound enzyme (HE), *i. e.*, more than one reaction path is possible. It has been pointed out by Segal, *et al.*,[11] that in such a case linear relationships, *e. g.*, those in Fig. 1, cannot be expected unless the system is in quasi equilibrium. As shown by Morales[12] this would also be a consequence of the assumption that the hydrogen ion is an independent ("non-competitive") modifier. Therefore, K_S and K_{H1} would represent equilibrium constants. From the consideration that k_2 must be small compared to the constant of the ionic reaction E − P_2 + H → HE-P_2 it would follow that this is also the case with K_{H2}.

Equilibrium treatment of the above reaction scheme, with the aid of the equations $v = k_1[HES] = k_2[E - P_2]$ and $E_{total} = E + HE + ES + HES + E\text{-}P_2 + HE\text{-}P_2$, shows that the relationship between v and S can be described by the original Michaelis equation

$$v_m = v + K_M \times v/S$$

where

$$V_m = k_1[E_t]/[(1 + K_{H1}/(H) + k_1(1 + [H]/K_{H2})/k_2] \quad (1)$$

and

$$K_M = K_S(1 + K_{H1}/[H]/[(1 + K_{H1}/[H] + k_1(1 + [H]/K_{H2})/k_2] \quad (2)$$

while

$$V_m/K_M = k_1[E_t]/K_S(1 + K_{H1}/[H]) \quad (3)$$

Written in the form

$$k_1 \times [E_t]/K_S = V_m/K_M + K_{H1} \times V_m/K_M \times [H]$$

equation 3 shows that a plot of V_m/K_M *versus* $V_m/K_M \times [H]$ (Fig. 1B) is linear with slope K_{H1}. The intercept with the ordinate equals $k_1 \times [E_t]/K_S$, which can be looked upon as the over-all rate constant for enzyme-glucoside formation since it is independent of k_2, the hydrolysis constant.

K_S is determined as follows: For the point of crossing of two curves at different *p*H in Fig. 1A it is found that

$$K_S = SH'H'' \times k_1/K_{H1} \times K_{H2}k_2 - S \quad (4)$$

where S, the substrate concentration, corresponds to the slope of a line through the origin and the point of crossing, and H' and H'' represent the two hydrogen ion concentrations. This formula results from equating two reaction rates at different *p*H, given by $v = V_m/(1 + K_M/S)$, while substituting equation 1 and equation 2 for V_m and K_M, respectively.[13] An intercept of the linear plot of $SH' \cdot H''$ *versus* $-S$ (Fig. 2A) equals K_S while the slope is $k_1/k_2K_{H1}K_{H2}$.

The constants K_{H2} and $k_2[E_t]$ are determined by subtracting two $1/K_M$ values (equation 2) at different *p*H. Through the use of equation 3 one finds

$$k_2[E_t]\Delta(1/K_M) = \Delta(V_m/K_M) + \Delta(H \times V_m/K_M)/K_{H2} \quad (5)$$

Thus the reciprocal (negative) slope of a plot of $\Delta(V_m/K_M)/\Delta(1/K_M)$ *versus* $\Delta(H \times V_m/K_M)/\Delta(1/K_M)$ equals K_{H2} and one intercept gives $k_2[E_t]$. The six possible combinations of 2 curves from the 4 curves in Fig. 1A correspond to the six points in Fig. 2B.

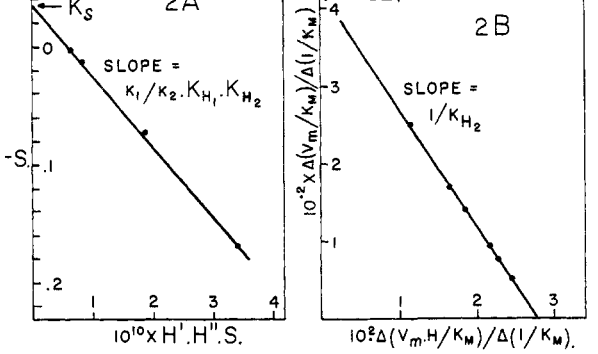

Fig. 2.—Part 2A. Determination of the dissociation constant (K_S) of the enzyme substrate Michaelis complex. The four substrate concentrations (S) are those at the points of crossing of the curves of Fig. 1A; H' and H'' are the corresponding hydrogen ion concentrations. Part 2B. Determination of the hydrogen ion dissociation constant (K_{H2}) of the enzyme-glucoside and of the enzyme-glucoside hydrolysis constant ($k_2[E_t]$). The six points correspond to the six possible combinations of two of the four curves of Fig. 1A.

KINETIC CONSTANTS DERIVED FROM THE ABOVE LINEAR PLOTS

K_{H1}, M	K_{H2}, M	K_S, M	$\frac{k_1}{[E_t]}$	$\frac{k_2}{[E_t]}$[a]	k_1/k_2
1.0×10^{-4}	6.5×10^{-5}	3.5×10^{-2}	16.5[b]	4.2[b]	3.9

[a] k_2 is not an elementary constant since it is a function of the water concentration. [b] μmoles/3 ml./15 min. at 29°; enzyme concentration unknown.

Discussion.—The fact that linear plots (Figs. 1B, 2A, 2B) are obtained on the basis of the postulates that were made and that the experimental data fit the curves (Fig. 1A) calculated from the pertaining kinetic constants does not give unequivocal proof that these postulates are correct. However, it can be stated that the proposed mechanism is at least consistent with the experimental data.

Such a change of K_M is not observed (Fig. 1A), which would suggest that the electronically inert glucose moiety of the substrate is oriented toward the ionizable site in the active center of the enzyme.

(11) H. L. Segal, J. F. Kachmar and P. D. Boyer, *Enzymol.*, **15**, 187 (1952).

(12) M. F. Morales, THIS JOURNAL, **77**, 4169 (1955).

(13) From $dv/dH = 0$ it is found that $H^2_{opt} = H'H''$, where H_{opt} is the optimum hydrogen ion concentration at substrate concentration S. Since $\log H_{opt} = (\log H' + \log H'')/2$ it follows that a plot v *versus* *p*H is symmetrical.

In this mechanism the formation of a Michaelis complex would not be necessary *a priori*. Apparent Michaelis behavior might be observed if the enzyme would react directly with the substrate in a bimolecular reaction $EH + S \rightarrow HE\text{-}P_2 + P_1$, where at high substrate concentrations the hydrolysis of the enzyme-glucoside might become rate limiting. However, in this case K_S would not be finite (Fig. 2A).

The observation that the pK of the essential ionizable group in the free enzyme and in the Michaelis complex is 4.0 as compared to about 4.2 in the enzyme-glucoside does not contradict the supposition that only one such group is involved. The small difference in pK could be accounted for by the covalent bond between glucose and a neighboring group in the enzyme. Unlike the preliminary adsorption of S in the Michaelis complex the formation of this bond might result in a shift of electrons toward the ionizable group, *i. e.*, in a weakening of its acidity. The magnitude of the pK suggests that it is a carboxyl group.

Cunningham recently has proposed a theory for the mechanism of action of hydrolytic enzymes.[2] This theory is based on consecutive "acylation" and "deacylation" reactions similar to glucoside formation and subsequent hydrolysis in our scheme. However, it is thereby assumed that, unlike the present case, the rates of both these reactions increase with decreasing hydrogen ion concentration from which it would follow that with increasing pH the rate approaches a maximum instead of displaying an optimum. The theory may be applicable to the case of chymotrypsin,[14] but without modification it could not explain the pH optimum of β-glucosidase.

(14) G. H. Dixon and H. Neurath, *J. Biol. Chem.*, **225**, 1049 (1956).

PALO ALTO, CALIF.

[CONTRIBUTION FROM WESTERN REGIONAL RESEARCH LABORATORY[1]]

Characterization of Coumestrol, a Naturally Occurring Plant Estrogen

BY E. M. BICKOFF, R. L. LYMAN, A. L. LIVINGSTON AND A. N. BOOTH

RECEIVED NOVEMBER 11, 1957

The estrogen coumestrol, previously isolated from ladino clover, has been characterized as 6′,7-dihydroxybenzofuro[3′,2′,-3,4]coumarin. Confirmation of this structure was accomplished by a series of progressive degradations involving (a) alkaline methylation to 2-(2′,4′-dimethoxyphenyl)-6-methoxybenzofuran-3-carboxylic acid; (b) decarboxylation by pyrolysis of the acid to 2-(2′,4′-dimethoxyphenyl)-6-methoxybenzofuran; (c) cleavage of the double bond by ozonolysis to form 4-methoxy-2-(2′,4′-dimethoxybenzoyl)-benzoic acid; and (d) final hydrolysis of the ester to 2,4-dimethoxy- and 2-hydroxy-4-methoxybenzoic acids.

Introduction

Following the isolation of an estrogenic compound from ladino clover,[2] preliminary investigations[3] resulted in the proposed structure (Fig. 1, IV), and the name "coumestrol" for the compound.

The presence of two free hydroxyl groups was confirmed by the formation of a diacetate and dimethyl ether derivative.[2] The empirical formula $C_{15}H_8O_5$ differed from that of a typical flavonoid only by the absence of two hydrogen atoms. Fusion studies yielded only resorcinol and β-resorcylic acid, giving an indication of the number and position of the hydroxyl groups on the two rings. The ultraviolet absorption spectrum of coumestrol differed markedly from the known estrogenic isoflavones, and was more characteristic of a flavone. However, comparison of coumestrol with a known flavone (7,2′,4′-trihydroxyflavone) having the required hydroxyl pattern indicated by the fusion products was sufficiently different to rule out flavones as a possibility.

That coumestrol might be a coumarin derivative was suggested[4] by the fact that its blue fluorescence was not appreciably affected by exposure to ammonia and that the bathochromic shift with alkali was much less than that expected from a flavone. A coumarin-type structure was demonstrated by methylation under alkaline conditions followed by saponification.[5] This reaction formed an *o*-methoxycarboxylic acid which gave an equivalent weight of 331 upon titration of the carboxyl group with methanolic potassium hydroxide solution. A naturally occurring 4-phenylcoumarin (dalbergin) had recently been isolated by Ahluwalia, *et al.*[6] How-

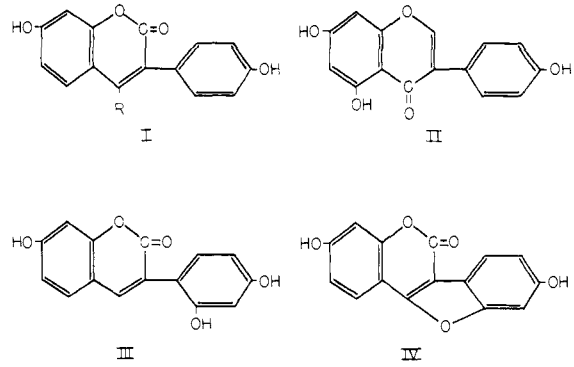

Fig. 1.—Structural formulas of Mentzer's estrogen (I), genistein (II), coumarin derivative related to coumestrol (III) and coumestrol (IV).

(1) Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture. Article not copyrighted.

(2) E. M. Bickoff, A. N. Booth, R. L. Lyman, A. L. Livingston, C. R. Thompson and G. O. Kohler, *J. Agr. Food Chem.*, in press.

(3) E. M. Bickoff, A. N. Booth, R. L. Lyman, A. L. Livingston, C. R. Thompson and F. DeEds, *Science*, **126**, 969 (1957).

(4) We are indebted to Prof. E. Jorgensen, University of California Medical School, for this observation.

(5) F. W. Canter and A. J. Robertson, *J. Chem. Soc.*, 1875 (1931).

(6) V. K. Ahluwalia and T. R. Seshadri, *ibid.*, 970 (1957).