[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

# Kinetics of the Acid and Enzyme Hydrolysis of *cis-trans* Enolic D-Glucosides and D-Galactosides of Methyl Acetoacetate[1]

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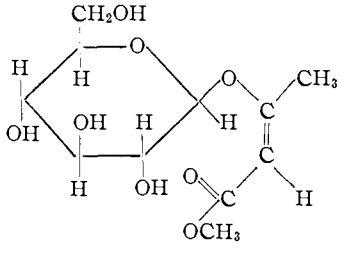
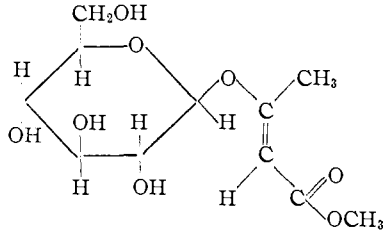
The nature of the glycosidic linkage in enolic glucosides and galactosides of methyl acetoacetate has been further characterized by a study of the acid- and enzyme-catalyzed hydrolysis of the *cis-trans*-forms of these compounds. The aglycon configuration has a profound effect on the rate of enzyme hydrolysis, but not necessarily on the enzyme affinity for the substrate. The enolic structure confers unusual acid lability on the glycosidic linkage. However, the rates of hydrolysis of the *cis*- and *trans*-forms do not differ widely.

Synthesis of *cis-trans*-isomer pairs of enol β-D-glycosides of methyl acetoacetate was realized in this Laboratory[2] using a modification of the Robertson method.[3] This consists of the use of catalytic amounts of quinoline or other amines along with silver oxide, the condensing agent in the Koenigs–Knorr glycoside synthesis. The β-D-glucopyranosides (I and II) are typical examples of these enolic glycosides. The β-anomeric configura-



I-*trans*



II-*cis*

tions and the enolic structure were established by chemical and physical means. Absolute configurations in the aglucon were assigned on the basis of observations indicating intramolecular association of the *trans*-isomer. This isomer (I) exhibited an abnormal molecular rotation of +12,100° in water as contrasted with molecular rotations of −5,000° to −25,000° common for β-D-glycosides, and showed an unusually large change in rotation with increase in temperature, the values tending toward the expected levorotatory values, an effect that was completely reversible. These abnormalities were compared with the similar properties of *o*-nitrophenyl β-D-glucopyranoside tetraacetate reported by Pigman[4] as being indicative of intramolecular association, and were cited as evidence of *trans* structure since inspection of Hirschfelder molecular models indicated that only the isomer having the structure designated *trans* could undergo intramolecular association.

The *trans*-isomer had a normal molecular rotation in dioxane (−18,900°) and in other non-polar solvents. This is contrary to the observation that polar solvents tend to minimize abnormalities in optical rotation (disrupt intramolecular associations), while solvents of low dielectric constant like dioxane tend to amplify these same abnormalities.[5] To rationalize these observations, a resonant structure stabilized by polar solvents was proposed.

This paper deals with the acid- and enzyme-catalyzed hydrolysis of the glucosides and galactosides of methyl acetoacetate, and the results further characterize the unusual nature of these enol glycosides.

The method for determining the rates of hydrolysis was based on the strong ultraviolet absorption of the glycosides due to the double bond conjugated with the carbomethoxy group.[2] This absorption disappears as the glycoside is hydrolyzed, permitting the course of hydrolysis to be followed spectrophotometrically. The method is very sensitive, and rates may be determined with solutions $2.5 \times 10^{-4}$ *M* in glycosides.

Enzymatic hydrolysis of the two galactosides showed that they were cleaved at greatly different rates. The *cis*, non-associated form was hydrolyzed rapidly and from the data was calculated an enzyme–substrate affinity comparable to that found for other β-D-galactosides and the same enzyme.[6] The plot of reciprocal velocity against reciprocal substrate concentration in Fig. 1, indicates a satisfactory agreement with theory.[7] The temperature effect observed is reasonable, the

TABLE I

ENZYMATIC HYDROLYSIS OF GALACTOSIDES

| Compounds | Temp., °C. | Maximal velocity moles/liter/30 minutes × $10^4$ | *Ks* (*M*) × $10^4$ |
|---|---|---|---|
| *cis*- | 25 | 1.8 | 5.1 |
| | 35 | 3.0 | 7.3 |
| *trans*- | 35 | *Ki*[a] | 0.75 |

[a] Obtained from the inhibition of the galactosidase activity on one concentration of the *cis*-form by varying concentrations of the *trans*-form (competitive inhibition is not proved).

(1) Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

(2) C. E. Ballou and K. P. Link, THIS JOURNAL, **72**, 3147 (1950).

(3) C. F. Huebner, S. A. Karjala, W. R. Sullivan and K. P. Link, *ibid.*, **66**, 906 (1944).

(4) W. W. Pigman, *J. Research Natl. Bur. Standards*, **33**, 129 (1944).

(5) T. M. Lowry, "Optical Rotatory Power," Longmans, Green and Co., New York, N. Y., 1935, p. 350.

(6) J. Lederberg, *J. Bact.*, **60**, 381 (1950).

(7) P. W. Wilson, "Kinetics and Mechanisms of Enzyme Reactions" in "Respiratory Enzymes," Burgess Publishing Co., Minneapolis, Minn., 1949

TABLE II

KINETICS OF THE HYDROLYSIS OF *cis–trans* METHYL ACETOACETATE GLYCOSIDES (0.01 *N* HYDROCHLORIC ACID)

| | Glycoside | Average $k \times 10^{4a,b}$ (min.$^{-1}$) at 64.5° | at 85.0° | $k_{trans}/k_{cis}$ at 64.5° | at 85.0° | Activation energy, cal./mole |
|---|---|---|---|---|---|---|
| I | *trans*-O-(β-D-Glucopyranosyl) methyl acetoacetate, m.p. 186–187° | 39.9 | 268 | 3.91 | 3.90 | 22,360 |
| II | *cis*-O-(β-D-Glucopyranosyl) methyl acetoacetate, m.p. 143–145° | 10.2 | 68.7 | | | 22,370 |
| III | *trans*-O-(β-D-Galactopyranosyl) methyl acetoacetate, m.p. 160–161° | 47.9 | 327 | 3.63 | 3.57 | 22,480 |
| IV | *cis*-O-(β-D-Galactopyranosyl) methyl acetoacetate, m.p. 188–189° | 13.2 | 91.5 | | | 22,700 |
| V | *trans*-O-(β-D-Glucopyranosyl) acetoacetic acid | 49.7 | | | | |

[a] Briggsian logarithms and minutes. [b] Average of two determinations of *K*. Mean deviations ranged from 0 to 3.57%, averaging 1.24%.

maximal velocity being approximately doubled by an increase of 10° (Table I).

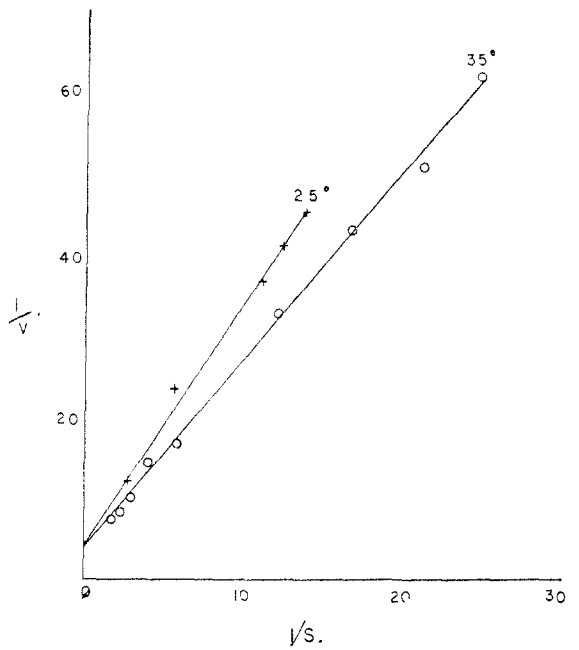



Fig. 1.—Enzyme hydrolysis of *cis*-O-(β-D-galactosyl)-methylacetoacetate; plot of reciprocal velocity, *v*, (millimoles/liter/30 minutes) against reciprocal substrate concentration, *S*, (millimoles/liter) at 25 and 35°.

The *trans*, or associated, form of the galactoside was apparently unattacked by the enzyme. From this one might conclude that it existed in a steric modification that could not combine with the enzyme. Such enzymatic specificity would support the postulate of an abnormal structure. However, when the *trans*-galactoside was introduced into the *cis*-galactoside–enzyme system a pronounced inhibition in the hydrolysis of the *cis*-form was observed, and the enzyme affinity for the *trans*-galactoside (*Ki*) was calculated to be about ten times the affinity (*Ks*) for the *cis*-form.[7]

Results of the acid-catalyzed hydrolysis of the glycosides are given in Table II. Although the compounds contain two acid labile groups, ester and glycoside, the latter is apparently much more sensitive to acid. That the results given in Table II are the rates for first-order hydrolysis of the glycosidic linkage is indicated by their conformation to first-order plots of log *C* against time, and by the fact that saponification of the ester followed by acid hydrolysis of the glycosidic linkage gives comparable results. Furthermore, hydrolysis of the ester does not affect the absorption spectra of the compounds in the manner observed. The decrease in ultraviolet absorption could only be due to hydrolysis of the glycosidic linkage.

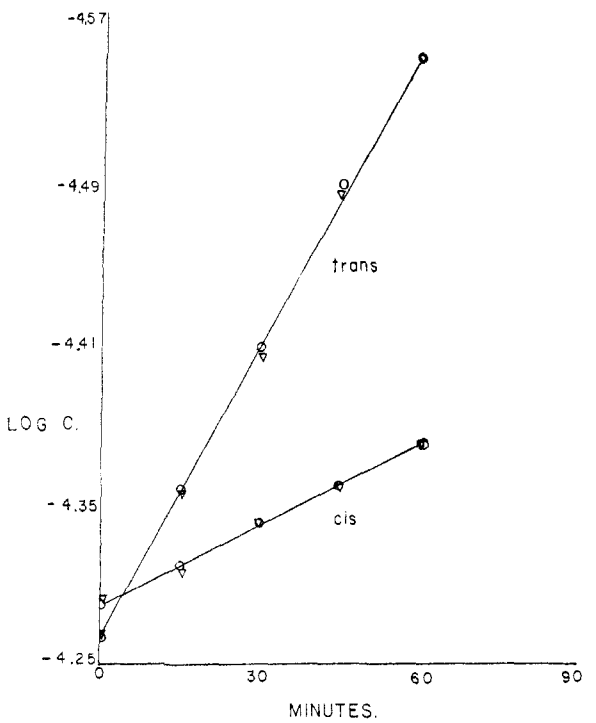



Fig. 2.—Acid hydrolysis of *cis*- and *trans*-O-(β-D-galactosyl) methyl acetoacetate at 64.5 ± 0.2° in 0.01 *N* hydrochloric acid, plot of log concentration against time in minutes.

From the table it is seen that the *trans*-forms are hydrolyzed almost four times faster than the *cis*-forms, although the activation energies are practically identical. This difference is probably due to steric effects associated with the *cis–trans* structures. The rates of acid hydrolysis are unusually high and indicate that these enolic glycopyranosides are of the order of stability of furanosides, a fact which may be related to their alkali lability.[2]

## Experimental

**Enzyme Preparation.**—The β-galactosidase was prepared from dried washed cells of *E. coli*, Strain K-12, according to Lederberg.[6] The aqueous extract of a 1% suspension of the ground cells was diluted 1:100 with *p*H 7.0 Clark and Lubs buffer (0.05 *M*).

**Enzyme Hydrolysis.**—The substrate was prepared approximately $10^{-3}$ *M* in *p*H 7 buffer (0.05 *M*). Aliquots of this standard solution were made to 12 ml. with buffer and 4 ml. of the stock enzyme solution was added to each (both

solutions having previously been brought to the temperature desired in a carefully regulated water-bath).

At appropriate times an aliquot of 4 ml. was removed from each reaction vessel and added to a tube containing 3 ml. of $2 \times 10^{-4}$ *M* mercuric chloride solution, which stopped the enzymatic action. The unhydrolyzed galactoside was estimated from the solution density at its absorption maximum[2] (232 or 236 mμ) in the Beckman DU spectrophotometer following dilution when necessary. Correction was made for absorption due to the enzyme and reagents. The velocity $v$ is expressed in millimoles per liter per 30 minutes; and the substrate concentration, $S$, in millimoles per liter. The results were plotted and the slope and intercept calculated by the method of least squares.

**Acid Hydrolysis.**—The experiments were run in a water-bath regulated to ±0.2°. To 25.0 ml. of 0.02 *N* hydrochloric acid preheated five minutes at bath temperature was added 25.0 ml. of a solution of approximately 140 mg. of glycoside per liter, preheated for the same period. At the end of one minute a 5.0-ml. sample was withdrawn and placed in 20.0 ml. of 0.1 *M* *p*H 7.0 Clark and Lubs buffer. Samples were removed at 15-minute intervals, so that five samples in all were obtained. Using a Beckman DU spectrophotometer, the ultraviolet absorption at the absorption maximum of each buffered sample was determined in a 1-cm. quartz cell against a blank composed of 10.0 ml. of 0.02 *N* hydrochloric acid, 10.0 ml. of water, and 80.0 ml. of 0.1 *M* *p*H 7.0 Clark and Lubs buffer. Duplicate hydrolyses of each isomer were performed. Optical density readings were converted to logarithms of concentration, plotted against time, and the linear relationship characteristic of first-order reactions was established. Slopes were derived statistically by the method of least squares and the average of two determinations for each compound was taken. Rates are expressed in Briggsian logarithms and minutes, according to custom in carbohydrate studies. Heats of activation were calculated from these average rates of hydrolysis using the integrated form of the Arrhenius equation.

**Acid Hydrolysis of the Saponified Glucoside (V).**—To a solution of 38.0 mg. of I in about 20 ml. of water was added 0.153 meq. of sodium hydroxide, and the solution was kept at room temperature (25°) for 45 minutes. An equivalent amount of hydrochloric acid was added and the solution was diluted to 100 ml. This treatment effects complete saponification of the methyl ester, as indicated by the change of the absorption coefficient to a constant value. (The optical rotation was followed on a more concentrated sample and came to a constant value in about the same length of time).

The determination and the calculation of the hydrolysis rates were conducted as described above. The extinction coefficient for the saponified glucoside was calculated from the average of the optical densities at zero time for the two runs, $\epsilon = 7.05 \times 10^3$ at 236 mμ.

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[CONTRIBUTION FROM THE GIBBS LABORATORY, DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY]

# The Association of Insulin. I. Preliminary Investigations

BY PAUL DOTY, MARTIN GELLERT AND BERNARD RABINOVITCH

A light scattering investigation of insulin solutions show that below *p*H 2.2 the monomer–dimer (molecular weight 12,000 and 24,000) equilibrium can be isolated. Data covering a fifty fold range in concentration can be fitted with a single equilibrium constant. At higher *p*H values tetramer and probably trimer exist. The tetramer species is dominant at *p*H 3.17 above a concentration of 0.3%. The effect of varying the ionic strength is illustrated. It is shown that earlier data on less highly purified insulin preparations may not be in conflict with the behavior just summarized. This behavior is in fair agreement with published osmotic pressure measurements but it is in disagreement with the current interpretation of sedimentation constants.

For more than a decade the molecular state of insulin in solution has been in dispute. Meanwhile great advances have been made in our knowledge of the internal structure of insulin and it has become increasingly evident that association and dissociation reactions of proteins in general deserve critical investigation by a variety of physical methods. Consequently we have undertaken an investigation of insulin solutions by light scattering methods.

Since the available data on the problem have been reviewed recently by several authors[1-3] our references to earlier work need not be comprehensive. Crowfoot's[4] X-ray studies in 1938 showed the unit cell molecular weight to be 37,600. Later revision of the moisture content changed this value to 36,000. The existence of trigonal symmetry led to the suggestion that three molecules of 12,000 molecular weight are arranged with this symmetry in the unit cell and may constitute the kinetic unit which exists in solution.

The determination of the mean size of the kinetic unit in solution has been the subject of numerous investigations using sedimentation and diffusion and osmotic pressure measurements. In neutral solutions molecular weights in the range of 36,000 to 46,000 are obtained,[3,5] the differences arising principally from the use of different values of the partial specific volume. Gutfreund[6] reports osmotic pressure molecular weights of 48,000 in this same range at concentrations of 0.5 to 0.8%. However, at lower concentrations dissociation was evident. Other experiments, mostly by these same workers, have shown that the degree of association is greatly decreased in acid solutions, in particular by decreasing the *p*H, the ionic strength and the dielectric constant. There is, however, no quantitative agreement on the dominant species present. Thus at about *p*H 2.5 and ionic strength 0.1 Oncley and Ellenbogen[3] consider insulin to be in the form of monomer (molecular weight 12,000), Pedersen[5] in the form of dimer while Fredericq and Neurath[2] propose the existence of a half-monomer unit when sodium dihydrogen phosphate is the added electrolyte. Gutfreund's osmotic pressure data indicate an average molecular weight of a dimer under these conditions. As conditions are changed to favor association there is agreement, except for

(1) F. Sanger, *Ann. Reports Chem. Soc., Long*, 283 (1948).

(2) E. Fredericq and H. Neurath, THIS JOURNAL, **72**, 2684 (1950).

(3) J. L. Oncley and E. Ellenbogen, *J. Phys. Colloid Chem.*, **56**, 85 (1952).

(4) D. Crowfoot, *Proc. Roy. Soc.* (*London*), **A164**, 580 (1938).

(5) K. D. Pedersen, *Cold Spring Harbor Symposium on Quantitative Biology*, **14**, 148 (1949).

(6) H. Gutfreund, *Biochem. J.*, **42**, 156, 544 (1948).