Analysis of a Crystalline Mutarotase from Bovine Kidney Cortex†

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ABSTRACT: Mutarotase (aldose-1-epimerase) was purified to homogeneity from bovine kidney cortex and crystallized. Amino acid analysis indicates a total of 336 residues with a content of glycine, valine, phenylalanine, and tryptophan (40, 30, 18, and 5 residues/mol, respectively) of about twice the average. Treatment with cyanogen bromide cleaves the enzyme into three large peptides indicating the presence of at least two interior methionine residues. Kinetic analysis, gel permeation, and equilibrium dialysis studies with phloretin ($K_I = 2.2 \times 10^{-5}$ M) and diethylstilbestrol ($K_I = 2.1 \times 10^{-5}$ M) indicate a single active center. The specific activity (26,500 mol of α-glucose converted to β min^{-1} mg^{-1}) corresponds to a catalytic coefficient of 0.99×10^6 min^{-1} at 25°. Titration with *p*-chloromercuribenzoate shows all four half-cystines to be reduced and necessary for activity. First-order rate constants for both enzyme inactivation and SH complexing measured spectrophotometrically were identical (0.363 min^{-1}) and were reduced equally (0.165 min^{-1}) by added substrate. *N*-Bromosuccinimide inactivates, reacting with 3/5-tryptophan residues in water and 5/5 in 8 M urea. *N*-Acetylimidazole acetylates 8/11-tyrosine residues. The acetylated enzyme is catalytically active.

Mutarotase (aldose-1-epimerase) is a widely distributed enzyme which catalyzes the mutarotation of glucose and of a limited number of aldohexoses and aldopentoses of related configuration (Keilin and Hartree, 1952; Bentley and Bhate, 1960). Previous studies have reported evidence that the enzyme may function in sugar transport in kidney and intestine (Keston,1954, 1964; Bailey *et al.*, 1964, 1970).

We have recently isolated the enzyme in crystalline form from bovine kidney cortex. This paper describes the chemical analysis of the purified enzyme and the modification of certain of its physical and catalytic properties following chemical derivatization of some of the amino acids in the enzyme protein.

Materials and Methods

Sodium *p*-chloromercuribenzoate and Tris (Ultra Pure grade) were from Mann Research Laboratories. α- and β-D-glucose, α-methyl D-glucoside, α-methyl D-mannoside, α-D-galactose, phloridzin, phloretin, diethylstilbestrol, and *N*-bromosuccinimide (NBS) were from Nutritional Biochemicals Inc. Polyacrylamide gel reagents were from Canalco Inc. and Eastman Organic Chemicals. [$^{14}C_1$]Monoethyldiethylstilbestrol was from Amersham-Searle. *N*-Acetylimidazole from Eastman Organic was recrystallized from benzene before use. Hydroxylamine hydrochloride and salts of the heavy metals were from Fisher Scientific.

Mutarotation was monitored at a wavelength of 546 nm using a Bendix automatic polarimeter (Model NPL) coupled to a Beckman 10-in. log linear recorder. For the standard enzyme assay, 36 mg of crystalline α-D-glucose (200 mesh) as substrate was dissolved in 12 ml of buffered enzyme solution (EDTA, 5 mM, pH 7.2) and the first-order rate constant for the mutarotation reaction was measured over a 10-min period. The units of enzyme activity (micromoles of α-glucose converted to β at optimum substrate concentration at 25°) were derived as described previously (Bailey *et al.*, 1967).

The enzyme was purified from bovine kidney cortex by a modification of previously described procedures (Bailey *et al.*, 1969) which increased the overall yield to 35%. The final product was crystallized from 60% saturated ammonium sulfate by the reverse solution technique of Jakoby (1968) and the purity was confirmed by ultracentrifugation analysis and by the presence of a single band during disc gel electrophoresis at three pH values (Figure 1). The final preparation had a specific activity of 26,500 units/mg of protein. Further details of the purification procedures are given elsewhere (Bailey *et al.*, 1973).

Amino Acid Analysis of Purified Mutarotase. Acid hydrolysates were prepared according to the procedure of Moore and Stein (1963), using pure samples of bovine kidney mutarotase (100 μg, 2500 units) and for varying periods (22, 44, and 72 hr) of hydrolysis. Performic acid oxidation was carried out according to the method of Hirs (1967). Analyses were performed using a Beckman Model Spinco 120 automatic amino acid analyzer. We are indebted to Dr. Walter Lovenberg, National Institute of Health, Bethesda, Md., for assistance with the amino acid analyses.

Determination of Tryptophan. Since tryptophan is destroyed by hydrolysis, this amino acid was determined separately using three different procedures. Spectrophotofluorimetric analysis was carried out by the method of Udenfriend (1962) using pure mutarotase and crystalline trypsin and chymotrypsin as controls and pure tryptophan solutions as standards. The spectrophotometric procedure of Beaven and Holiday (1952) was used to measure both tryptophan and tyrosine content, utilizing the shift of the ultraviolet (uv) absorption peak of tyrosine in alkaline solution due to the ionization of the phenolic hydroxyl group.

Tryptophan content was also measured by the method of Spande and Witkop (1967). This procedure utilizes the shift in the uv absorption peak due to conversion to the oxindole following oxidation by *N*-bromosuccinimide. The purified enzyme was titrated with aliquots of *N*-bromosuccinimide following the process at 280 nm until the absorbancy reached

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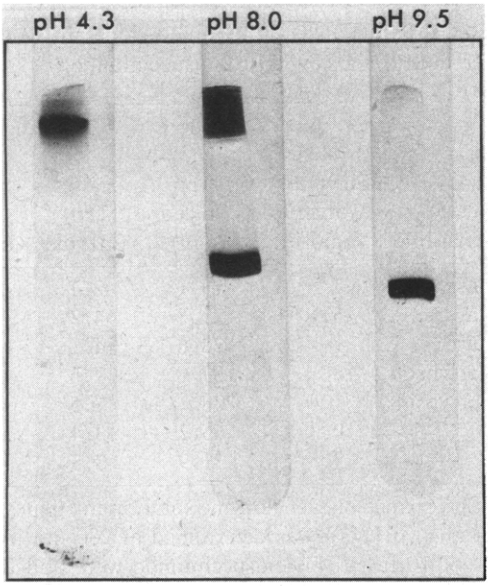

FIGURE 1: Electrophoresis of purified bovine kidney mutarotase in polyacrylamide gel at three pH values. The homogeneity of the crystalline enzyme preparation was demonstrated by electrophoresis at pH 9.5, 8.0, and 4.3. Aliquots, 20 μl (20 μg), of the purified enzyme were layered onto the gel columns in 20% sucrose. A current of 3 mA/column was applied and terminated when the tracking dye reached the bottom of the gels. Gels were fixed in acetic acid (12%), stained with Coomassie Blue dye, and destained in acetic acid (7%). The R_F values determined from the ratio of the distance traveled by the protein to the distance traveled by the solvent front were as follows: pH 9.5, 0.50; pH 8.0, 0.37; and pH 4.3, 0.0, indicating that the isoelectric point is close to pH 4.3.

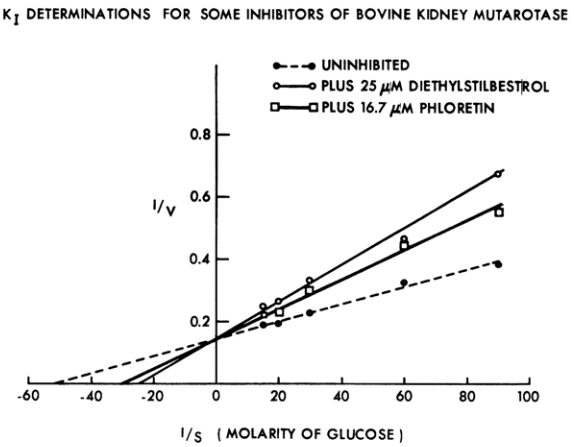

FIGURE 2: Determination of enzyme inhibitor dissociation constants for phloretin and diethylstilbestrol. The influence of increasing substrate concentration on the velocity of the mutarotase-catalyzed reaction in the presence and absence of inhibitors was determined. Pure mutarotase (54 units) was assayed polarimetrically with 36 mg of α-D-glucose and increasing amounts of equilibrium D-glucose. The total assay volume was 12 ml (5 mM EDTA, pH 7.2) and temperature was 25°. Each assay was repeated in the presence of 16.7 μM phloretin or 25 μM diethylstilbestrol. The net mutarotational rate constants (Δk) were converted to velocities and the data plotted reciprocally by the method of Lineweaver and Burk. From the intercepts on the abscissa of the inhibited and uninhibited plots, the enzyme–inhibitor dissociation constant, K_I, for each inhibitor was calculated from the equation $1/v = (K_m/V_{max})[1 + (1/K_I)](1/S) + (1/V_{max})$. With a K_m of 19 mM for D-glucose, the K_I for phloretin was 22 μM and for the diethylstilbestrol it was 21 μM. Since each plot intercepted the ordinate at the same point ($1/V_{max}$) the inhibitions were competitive.

a minimum. The experiment was then repeated in 8 M urea to determine tryptophan residues buried in the interior of the enzyme.

Oxidation Rates of Free Sulfhydryl Groups and Protection by Substrate. The spectrophotometric titration method of Boyer (1954) which utilizes the shift in the uv absorption of *p*-chloromercuribenzoate when it combines with an –SH group was employed. The reagent was standardized either by measurement of the absorbancy at 233 nm or alternatively by titration with pure solutions of mercaptoethanol or L-cysteine. Microliter aliquots of *p*-chloromercuribenzoate (1 mM) were added to quartz cuvets, one of which was a blank and the other containing pure mutarotase solution (2000–8000 units, 80–320 μg) in Tris buffer (10 mM, pH 7). The absorbancy at 250 nm was measured 15 min after each addition and an aliquot was removed and assayed for remaining enzyme activity. The rate of reaction of the SH groups with *p*-chloromercuribenzoate was also measured in separate experiments by adding a twofold excess of *p*-chloromercuribenzoate and following the increase in absorbancy at 250 nm with time.

Measurement of Enzyme–Inhibitor Binding Sites. These were measured in two ways using the competitive inhibitors phloridzin and diethylstilbestrol. In the first procedure the amount of enzyme–inhibitor complex was determined by the gel filtration chromatography method of Hummel and Dreyer (1962). The column and the eluting buffer were saturated with phloretin so that the enzyme–inhibitor complex was formed in the excluded volume. The absorbance of phloretin at 320 nm was monitored and fractions were collected and assayed for mutarotase activity. The number of binding sites per mole of enzym was derived using the dissociation constant of the enzyme–I loretin complex as measured in separate kinetic studies o enzyme inhibition as described in Figure 2.

The number of binding sites was also determined by the equilibrium dialysis procedure using phloretin or ^{14}C-labeled diethylstilbestrol as described in Table III.

Acetylation and Deacetylation of the Enzyme. The purified enzyme (1 mg) was acetylated using *N*-acetylimidazole according to the procedure of Riordan and Vallee (1967). The modified enzyme was assayed for ability to catalyze the mutarotation of glucose and a uv absorption scan was made on the solution. Deacetylation was carried out and the O-acetylated tryosine content of the enzyme was obtained as described in Figure 3. Crystalline trypsin was carried through the same procedure as a control protein of known tyrosine content.

Results

Amino Acid Composition. The uncorrected values for serine, threonine, and tyrosine were extrapolated to zero time to correct for losses during hydrolysis, and the total was corrected for water of hydrolysis. Amide nitrogen as ammonia was not determined. The molecular weight of mutarotase (37,000) was determined both by sucrose density gradient ultracentrifugation and electrophoresis in sodium dodecyl sulfate–polyacrylamide gels agaist standard proteins (Mulhern *et al.*, 1973). A value of 36,400 was calculated from the amino acid composition given in Table I.

The amino acid ratios were multiplied by the ratio of the resulting value to the molecular weight and rounded off to integral values. The half-cystine content was determined from the cysteic acid content of the performic acid oxidized sample. The four residues per mole obtained are in excellent agreement with the average figure of 3.92 SH groups/mol obtained by titration with *p*-chloromercuribenzoate.

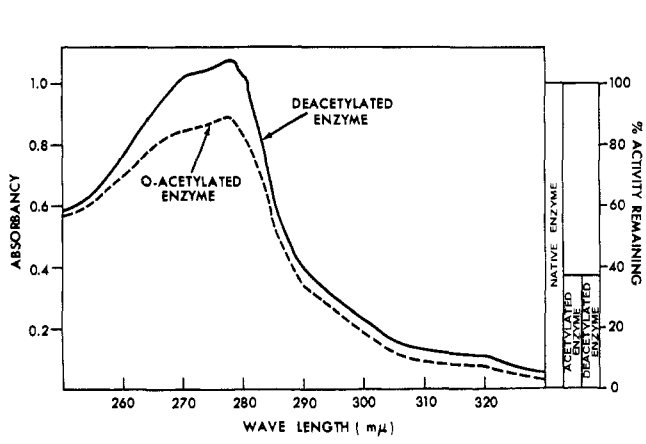

FIGURE 3: Uv absorption spectrum of O-acetylated mutarotase. A solution of sodium barbital buffer (1.4 ml, 20 mM, pH 7.5) containing 14,000 units (53 μg) of pure bovine kidney mutarotase was acetylated by reacting with 20 mg of *N*-acetylimidazole for 1 hr at 25°. Excess reagent was removed by ultrafiltration against barbital buffer. The ultraviolet spectrum of the final solution was measured against barbital buffer. Hydroxylamine (2 M, pH 7.5) was added to the protein and buffer solutions (final NH_2OH concentration was 45 mM). The absorbance at 278 nm was followed with time until a maximum value was obtained (30 min after the addition of NH_2OH). The absorption spectrum of the protein solution was scanned again and the absorbancies corrected for volume changes. From the molarity of mutarotase present and the net change in absorbance at 278 nm, the number of tyrosine residues acetylated per enzyme molecule was calculated according to Riordan and Vallee (1967). A total of 7.8 tyrosine residues were acetylated. Activities of the original enzyme, acetylated enzyme, and deacetylated enzyme were determined. The results suggest that tyrosine is not involved in the active site of mutarotase.

TABLE I: Amino Acid Composition of Bovine Kidney Mutarotase.[a]

Amino Acid	Mol %	g %	Residues/ Molecule
Lysine	4.16	4.42	14
Histidine	2.97	3.76	10
Arginine	3.57	5.16	12
Aspartic	10.71	11.38	36
Threonine	6.54	6.11	22
Serine	5.95	4.78	20
Glutamic	11.90	14.19	40
Proline	4.46	4.00	15
Glycine	11.90	6.27	40
Alanine	6.56	4.29	22
Valine	8.92	8.17	30
Isoleucine	2.38	2.48	8
Leucine	8.63	9.01	29
Tyrosine	3.27	4.93	11
Phenylalanine	5.35	7.27	8
Half-cystine	1.19	1.13	4
Tryptophan	1.48	2.25	5
Methionine			2

[a] The amino acid composition of mutarotase was determined on acid hydrolysates as described in Methods. The methionine content was measured independently by the cyanogen bromide cleavage procedure. Tryptophan was determined separately by the spectrofluorimetric analysis described in Methods.

TABLE II: Oxidation of Tryptophan Residues in Mutarotase and Reference Proteins by *N*-Bromosuccinimide.[a]

Enzyme Sample	Enzyme Concn (μmol)	Trp Oxidized (μmol)	Trp: Enzyme	NBS[c] Consumed: Trp Oxidized
Mutarotase	7	19.1	2.7	7.9
Mutarotase[b]	5.1	21.5	4.2	6.8
Mutarotase[b]	7	28.6	4.1	5.2
α-Chymotrypsin	18	137	7.6	2.9
α-Chymotrypsin[b]	31.4	238	7.5	2.7
Trypsin	19.3	65.2	3.4	3.1
Trypsin[b]	9.5	31.9	3.3	3.1

[a] The absorbance of each enzyme solution in 50 mM sodium acetate buffer (pH 4.0) was determined at 280 nm. Aliquots (2–10 μl) of 10 mM *N*-bromosuccinimide were added to the protein solution with rapid mixing and the absorbance redetermined after 5 min. The addition of reagent was continued until the absorbance at 280 nm was at a minimum. The experiment was repeated in the presence of 8 M urea. From the decrease in absorbance at 280 nm, the molarity of tryptophan oxidized was calculated according to Spande and Witkop (1967). Dividing the molarity of the ratio of tryptophan by the molarity of protein yields the molar ratio of tryptophan to protein. The difference of this ratio with and without 8 M urea indicates the number of "free" and "buried" tryptophans. in the molecule. The ratio of *N*-bromosuccinimide:tryptophan indicates the selectivity of the oxidation. A low ratio means that primarily tryptophan residues are being oxidized; a high ratio indicates other residues are being oxidized. [b] Plus 8 M urea. [c] *N*-Bromosuccinimide.

The values obtained for methionine content were variable averaging only about 0.5 mol/mol of enzyme. An independent measure of methionine content was obtained by reaction with cyanogen bromide according to the procedure of Gross and Witkop (1962). The products were analyzed by electrophoresis on sodium dodecyl sulfate gels according to the procedure of Weber and Osborn (1969) and three large peptide fragments were obtained. This result indicates that the native enzyme probably contains at least two methionine residues at which cleavage by cyanogen bromide occurs.

Values of 4.55 mol of tryptophan/mol of enzyme and 8.9 mol of tyrosine/mol of enzyme were obtained based upon the shift in the ultraviolet spectrum of the enzyme in alkali. Spectrophotofluorimetric analysis indicated 5.1 mol of tryptophan. Under the same experimental conditions values of 7.7 for chymotrypsin and 4.3 for trypsin were obtained which are in good agreement with the literature values of eight and four residues of tryptophan/mol, respectively.

The oxidation of tryptophan residues using *N*-bromosuccinimide in dilute buffer and in 8 M urea to expose buried groups is summarized in Table II together with the values obtained for the proteins used as a control for the procedure which are in good agreement with the literature. In aqueous buffer an average of 2.7 mol of tryptophan/mol of enzyme was oxidized and in 8 M urea the value increased to an average of 4.2.

Titration of SH Groups and Enzyme Activity. It was found

that a number of heavy metal ions inhibited the pure enzyme although in varying degrees. (At a concentration of 1.3 μM, Ag^+ and Hg^{2+} inhibited the enzyme completely. At concentrations of 13.3 μM approximately 80% inhibition was produced by Cu^{2+} and about 20% by Zn^{2+}, Co^{2+}, Pb^{2+}, and Cd^{2+}. No inhibition was observed for Cr^{3+}, Mn^{2+}, Ni^{2+}, Fe^{3+}, Al^{3+}, or UO_2^{2+}.) The enzyme was also inhibited by micromolar concentrations of *p*-chloromercuribenzoate which was used to determine the sulfhydryl group content of the enzyme.

During the spectrophotometric titration of SH groups there was a direct correspondence between loss in enzyme activity and sulfhydryl complexing with all activity lost when about four sulfhydryl groups were allowed to react. The free SH group content was determined under a number of different reaction conditions and an average value of 3.92 mol/mol of enzyme, based upon a mol wt of 37,000, was obtained. The presence of substrate (D-galactose) at close to saturation concentrations (125 mM) had no effect on the number of titratable SH groups but did influence the rate of complexing and inactivation considerably (Figure 4). Inactivation and reaction of SH groups measured spectrophotometrically followed first-order kinetics at a uniform rate to less than 10% residual activity, the first-order rate constants for both SH complexing and inactivation being the same (0.36 ± 0.003 min^{-1}). When substrate was added the rate of both processes was reduced by about 50% to 0.165 ± 0.003 min^{-1}.

Acetylation of the Enzyme. The uv absorption spectra of acetylated and deacetylated mutarotase shown in Figure 3 indicated that 7.8 mol of tyrosine was acetylated/mol of enzyme. Using the reference protein trypsin a value of 6.7 mol/mol was obtained which agrees well with the literature value of Riordan *et al.* (1965), who obtained 6–7 mol for trypsin. The acetylated enzyme retained about 36% of the activity of the native enzyme, which was not increased by deacetylation with hydroxylamine.

Number of Binding Sites per Molecule of Enzyme. The elution pattern of pure bovine kidney mutarotase chromatographed on Sephadex G-25 in buffers saturated with the competitive inhibitor phloretin is shown in Figure 5. The increase in phloretin concentration over background which represents enzyme-bound phloretin parallels the mutarotase activity. On the average 0.43 nmol of phloretin was bound per 1000 units (1 nmol) of enzyme. Based on the K_I of 22 ± 1.9 μM for phloretin measured in competitive inhibition studies (Figure 2) and the average measured background concentration of phloretin (17.16 μM), the enzyme would be 43% saturated by inhibitor. The results thus indicate one binding site/mol of mutarotase. The results of the equilibrium dialysis studies (Table III) with phloretin as binding solute gave 0.96 and 0.83 mol/mol of enzyme at two different phloretin concentrations, and with [^{14}C]diethylstilbestrol as binding solute the values were 0.86 and 0.76 mol/mol of enzyme, respectively. Both these values were obtained by calculation of the theoretical binding from the independent measurement of the K_I of the enzyme–inhibitor measured in separate kinetic studies (Figure 2).

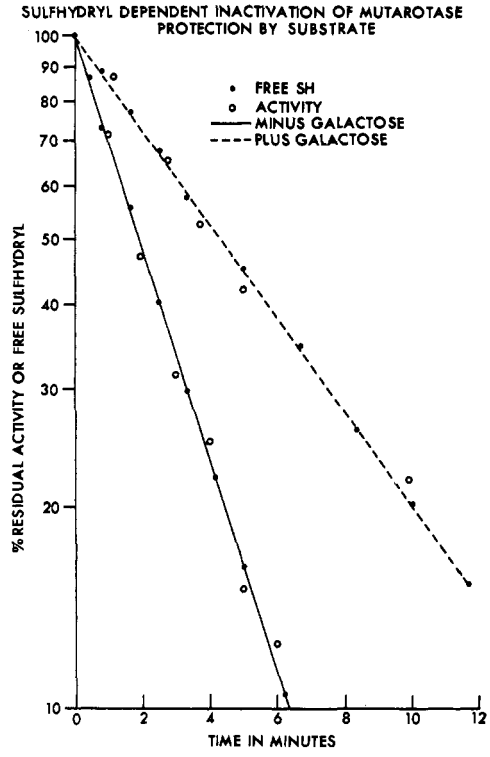

FIGURE 4: Sulfhydryl-dependent inactivation of mutarotase protection by substrate. The absorbance of a solution of pure bovine kidney mutarotase in 10 mM Tris-HCl (pH 7.0) was balanced at 250 nm against the same buffer in a Cary Model 15 recording spectrophotometer. At zero time 10 μl of *p*-mercuribenzoate (two-fold molar excess of reagent to free sulfhydryl) was added to both cuvets. An aliquot of the sample solution was removed for enzyme assay and the increase in absorbance at 250 nm followed with time. At the indicated times the separate aliquot was assayed for residual activity by means of the standard mutarotase assay. The entire procedure was repeated in the presence of 125 mM D-galactose. The first-order rate constants for inactivation and sulfhydryl complexing were the same, 0.363 ± 0.003 min^{-1} in the absence of substrate. When substrate was added, the rate of both processes was reduced to 0.165 ± 0.003 (average of duplicate experiments).

Discussion

The two competitive inhibitors used for determination of binding sites were chosen specifically for their low dissociation constants of the order of 10^{-5} M. In order to observe a significant increase in the concentration of bound solute over free solute, the concentration of enzyme must be of the same order of magnitude as the dissociation constant of the enzyme–solute complex. D-Fucose, the substrate with highest affinity for mutarotase, has a K_m of 2 mM (Bailey *et al.*, 1969). With an enzyme concentration of 400,000 units/ml an increase in substrate concentration on the side of the membrane containing the enzyme of only 10% could be expected. The nature of the enzyme–substrate interaction therefore precluded the use of substrate binding to determine the number of active sites in the mutarotase molecule. Since both phloridzin and diethylstilbestrol are simple competitive inhibitors it seems reasonable to equate the finding of one binding site for each of these compounds with the presence of only a single active center in the mutarotase molecule.

The agreement between half-cystine content measured by amino acid analysis and the titratable free sulfhydryl groups indicates that disulfide bridges are absent in the native molecule. The linearity of the rate curves illustrated in Figure 4 indicates that all the SH groups are accessible to the aqueous surroundings. Most of the tyrosine residues appear to be free and not buried. Spectrophotometric analysis in dilute alkali measures those tyrosines which ionize normally (pK values of 9.5–10.5) (Beaven and Holiday, 1952; Wetlaufer, 1962). A value of 8.9 residues/mol was obtained for mutarotase using this method. O-Acetylation with *N*-acetylimidazole also indi-

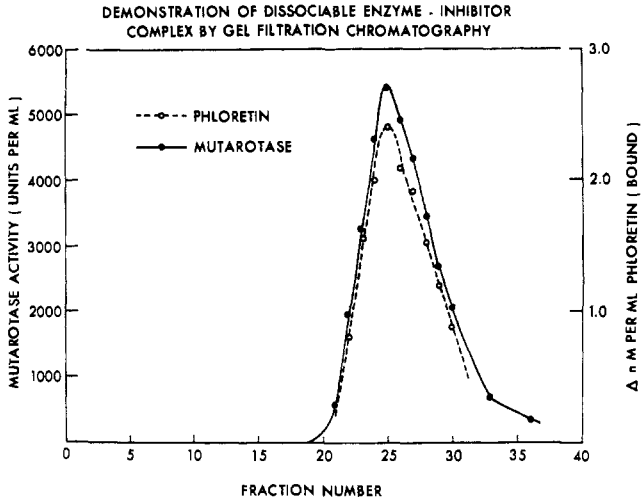

FIGURE 5: Demonstration of dissociable enzyme–inhibitor complex by gel filtration chromatography. A 0.55 × 100 cm column was packed with Sephadex G-25 (coarse grade) and equilibrated with 20 μM phloretin in EDTA buffer (5 mM, pH 7.2). An aliquot (0.5 ml) of pure bovine kidney mutarotase, representing 38,000 units of activity and 38 nmol of protein, was prepared in the same solution and placed on top of the bed. The enzyme was eluted with the same phloretin buffer at a flow rate of 20 ml/hr. The effluent was passed through a micro flow-through cuvet (1.0-cm path length) and the absorbance at 320 nm was monitored with a Beckman DB-G spectrophotometer and recorder. Phloretin absorbs strongly at 320 nm. Fractions of 0.5 ml were collected and assayed for mutarotase activity. The absorbance prior to elution of the enzyme was extrapolated through the region of elution of the enzyme and used as a base line to calculate the concentration of free phloretin. The increase in absorbance above this base line was also converted to phloretin concentration (Δ nmol per ml) and represents enzyme-bound inhibitor. The base-line optical density of the buffer was 0.250 and the increase due to enzyme-bound phloretin was approximately 15% of this value. There was an average of 0.4 nmol of phloretin bound per nmol of enzyme (1000 units of activity). From the free phloretin concentration (17.2 μM) and the K_I for phloretin (22 μM) it can be calculated that the enzyme should be 43% saturated with inhibitor. The results indicate one binding site for phloretin per molecule of mutarotase.

TABLE III: Measurement of Number of Binding Sites per Mole of Mutarotase by Equilibrium Dialysis.[a]

Concn of (μM)				
Enzyme	Free Solute	Bound Solute	Predicted Bound	No. of Binding Sites
		Diethylstilbestrol as Binding Solute		
25	5.6	4.5	5.2	0.86
25	22.1	9.4	12.8	0.73
		Phloretin as Binding Solute		
18.4	18.2	8.0	8.3	0.96
18.4	31.3	8.9	10.7	0.83

[a] On one side of an equilibrium dialysis chamber was placed 1 ml of a concentrated solution of pure kidney mutarotase; on the other side, 1 ml of a competitive inhibitor (phloretin or [^{14}C]diethylstilbestrol) dissolved in EDTA buffer (5 mM, pH 7.2). After equilibration was accomplished the concentrations of the components on both sides were determined. Enzyme activity was measured by the standard assay and converted into concentration using an equivalence of 1000 units/nmol of mutarotase. Phloretin concentration was determined by absorbancy at 320 nm (ε 1.25 × 10^4). Radioactive diethylstilbestrol was determined by liquid scintillation counting techniques. The difference in inhibitor concentration between the two sides of the chamber at equilibrium represented enzyme-bound inhibitor. From the concentration of free inhibitor, the K_I of the inhibitor, and the enzyme concentration, the predicted amount bound (based on one binding site per molecule of enzyme) was calculated. The ratio of actual to predicted bound solute therefore represents the number of binding sites per molecule of enzyme.

cated approximately eight residues/mol. Riordan *et al.* (1965) have examined the method in detail and have used the relative number of O-acetylated tyrosine residues in the presence and absence of denaturing agents such as 8 M urea as a criterion for distinguishing between free and buried tyrosines. Amino acid analysis of the enzyme indicates that there are 11 tyrosines/mol, hence, at least eight of these appear to be close to the surface. The importance of these to the enzyme catalysis is probably marginal since 36% reactivity was retained when all were acetylated. The fact that deacetylation did not increase the activity also suggests that the loss in activity upon acetylation was probably nonspecific and not directly related to acetylation of the tyrosines *per se*.

Some of the tryptophan residues on the other hand appear to be buried. Spectrophotofluorimetric analysis indicated five residues/mol of mutarotase. Only 2.7 mol could be oxidized with *N*-bromosuccinimide in aqueous solution whereas 4.2 residues/mol were accessible in 8 M urea. The overall results taking into account the limits of the experimental procedures suggest that three residues of tryptophan are free and two are buried. An estimate of the probable correctness of these limits can be seen by comparing the results obtained with the standard proteins, trypsin, and chymotrypsin with literature data given in Table II.

Interpretation of the sulfhydryl-dependent inactivation of mutarotase and the influence of substrate on the rate of inactivation is complicated. The results indicate that bovine kidney mutarotase is a "sulfhydryl" enzyme as apparently are all mutarotases so far isolated, but the problem is to explain the observation that saturating amounts of substrate reduce both the rates of enzyme inactivation and reaction of all four sulfhydryl groups to the same extent. The possibility that all four SH groups are involved in catalysis in an enzyme which has only one binding site is unusual and is difficult to justify on the basis of a simple model of the active center. It seems possible that the SH groups may not be involved directly in catalysis but that in the presence of substrate, the enzyme may undergo some type of conformational change which renders them less accessible to *p*-chloromercuribenzoate. Some preliminary evidence of conformational changes in the enzyme during catalysis of the mutarotation reaction has been obtained (Kusiak and Bailey, 1972) and is described in a following paper.

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Phosphorylation of Rhodopsin in Bovine Photoreceptor Membranes. A Dark Reaction after Illumination†

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ABSTRACT: We have found protein kinase activity in purified bovine rod outer segments (ROS). This activity requires Mg^{2+} and, as assayed with histones or protamine, is light independent. Rhodopsin, presumably the normal substrate, must be bleached before it can be phosphorylated. The kinase can be extracted from ROS fragments, demonstrated in the extract, and added back to restore the original light-dependent phosphorylation of rhodopsin. This reaction has not been demonstrated *in vivo*. A hypothetical mechanism is discussed in which phosphorylation blocks ionic channels which have been opened in response to light absorption and reduces the light sensitivity of the rod cell.

The retinal rod cell is a specialized neuron containing a receptor system triggered by light. The receptor molecule, rhodopsin, can be isolated relatively easily in amounts that are useful for biochemical and protein chemical studies. Thus, the rod is a useful model system for the study of membrane receptors.

The outer segment (a modified cilium) is the photodetector unit of the rod cell. The rat ROS,[1] for example, contains *ca.* 4×10^7 rhodopsin molecules densely packed in about 1000 parallel "disks" (Penn and Hagins, 1972). The disks are flattened, closed sacs containing primarily rhodopsin and phospholipids in their membranes. Their internal volume is reported to be high in Na^+ concentration (Govardovskiĭ, 1971).

The light sensitivity of the visual system can adapt so as to permit it to function over a wide range of light intensities, perhaps as much as nine orders of magnitude (Rushton, 1969). Two sorts of "light adaptation" can be observed. The one involves changes in the ability to detect, for example, intensity differences between a test spot and a background; this "increment threshold," as it is called, depends on the intensity of the background. Relatively low light intensities are used and the changes occur rapidly. This type of light adaptation presumably involves retinal processing (Rushton, 1969). The other sort of light adaptation involves slow, long-term changes in the sensitivity of the visual system and presumably occurs at the level of the receptor (Dowling and Ripps, 1972; Hodgkin, 1972; Hagins, 1972; Penn and Hagins, 1972). A fully dark adapted rod can, upon absorption of a single photon, respond and trigger the bipolar cell with which it synapses (Hecht *et al.*, 1942; Hagins *et al.*, 1970; Dowling and Ripps, 1972). On the other hand, the threshold and response–stimulus intensity function of skate rods can be shifted by several orders of magnitude in response to a steady background (Dowling and Ripps, 1972). Excessive triggering has also been observed to result in the desensitization of other receptor systems such as those involving the acetylcholine receptor (Thesleff, 1970). The molecular basis of these phenomena is not clear at this time.

It is generally accepted that the primary effect of light absorption by rhodopsin is the isomerization of the 11-*cis*-retinal chromophore to the all-trans form (Wald, 1968). Other changes then occur with, at least *in vitro*, the eventual formation of free *all-trans*-retinal and opsin (Wald, 1968). This process is called bleaching. The original light sensitivity of rhodopsin is restored when, through a cycle of reactions, 11-*cis*-retinal is again linked to the protein. Little is known about other chemical reactions in this cycle.

We have found, as described in a preliminary report (Kühn

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[1] Abbreviation used is: ROS, rod outer segment.