# Kinetic Studies of Bovine Carbonic Anhydrase Catalyzed Hydrolyses of Para-Substituted Phenyl Esters[*]

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ABSTRACT: The esterase activity of bovine carbonic anhydrase is examined with respect to a series of para-substituted phenyl acetate esters. For each ester a detailed pH–rate profile was obtained in 10% (v/v) acetonitrile at 25.0° over the pH range 6–10.5. In every case, the pH–activity curves are sigmoid, the esterase activity being very small below pH 6.5 and rising to a high level around pH 9; the inflection points for all the esters lie at pH 7.4. The esterase activity is completely inhibited by the binding of 1 molecule of sulfonamide. The observed k_{enz} values follow the order $p\text{-}NO_2 \gg p\text{-}C_6H_5 > p\text{-}Cl > p\text{-}i\text{-}Pr$, $p\text{-}Et$, $p\text{-}Me$, $p\text{-}H > p\text{-}MeO > p\text{-}COO^-$. The effects of the para substituents X on the free energy of the hydrolysis process are qualitatively factored, in operational terms, into hydrophobic, π, and electronic, σ, components. The value of k_{enz} could be approximated by the relation $\log k_{enz} = 2.31 + 0.49\pi + 1.80\sigma^-$.

Carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) (CA[1]) was the first example of a zinc-containing metalloenzyme (Keilin and Mann, 1940). The primary reaction catalyzed

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$
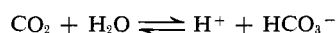
by the enzyme is readily reversible at physiological pH and proceeds at an appreciable rate even in the absence of the enzyme. Until the mid 1960's it was thought that the most remarkable property of CA was its absolute specificity with respect to the reversible hydration of carbon dioxide. However, Pocker and Meany (1964, 1965a,b) discovered that BCA and HCA would also catalyze the hydration of acetaldehyde. This observation has prompted numerous attempts to find additional examples of catalytic versatility. The enzyme was found to catalyze the reversible hydration of a number of aldehydes (Pocker and Meany, 1967a,b; Pocker and Dickerson, 1968), the dehydration of 2,2-dihydroxypropionate to pyruvate anion (Pocker and Meany, 1970), and the irreversible hydrolysis of a number of activated esters (Pocker et al., 1965; Pocker and Stone, 1965, 1967, 1968a,b,c; Pocker and Storm, 1968). Other investigators have also found the esterase activity to be very useful in the kinetic analysis of erythrocyte CA function (Tashian et al., 1964; Armstrong et al., 1966; Duff and Coleman, 1966; Thorslund and Lindskog, 1967; Verpoorte et al., 1967; Kaiser and Lo, 1969). More recently, we have delineated the kinetics and characterized the specificity of erythrocyte carbonic anhydrase with respect to the enzyme-catalyzed hydrolysis of pyridyl acetates (Y. Pocker and N. Watamori, 1971) and of pyridyl carbonates (Pocker and Guilbert,[2] 1972). In the present study, we report on an attempt to elucidate the structure–activity relationships in CA catalysis through a comparative analysis of substituent effects on (a) sulfonamide inhibition and (b) phenyl acetate hydrolysis.

The general approach for the formulation of extrathermodynamic relationships has been extensively analyzed for nonenzymatic reactions by Leffler and Grunwald (1963). We were ourselves impressed not so much by the usefulness of such relationships per se, as by their ability to detect changes in the rate-limiting step (Noyce and Snyder, 1959; Kirsch and Jencks, 1964). However, for enzymatic processes, the problem is much more complex. In particular, hydrophobic forces become extremely important and must play an important role in any model (Bender, 1962; Hennrich and Cramer, 1965; Van Etten et al., 1967; Pocker and Storm, 1968; Hansch, 1969). Characteristically, carbonic anhydrase shows a much greater sensitivity to the structure of the ester than does the hydroxide ion reaction as shown by the fact that phenyl acetate is a very poor substrate indeed compared to p-nitrophenyl acetate (Pocker and Stone, 1968b). The experiments reported here were carried out in an attempt to delineate in some detail the kinetic characteristics associated with the esterase activity of carbonic anhydrase.

Experimental Section

Materials

Substituted Phenols and Phenyl Acetate Esters. The following phenols were obtained from commercial sources: phenol (Mallinckrodt Chemical Co.); p-ethyl-, p-chloro-, p-phenylphenol, and p-hydroxybenzoic acid (Aldrich Chemical Co.); p-methoxy-, p-methyl-, and p-isopropylphenol (K & K Laboratories); p-nitrophenol (Matheson Co.); and p-tert-butylphenol (Eastman Kodak). With the exception of p-nitrophenol these compounds were first purified by fractional distillation at 0.1 mm using a heated Vigreux column and a hot water condenser; all phenols were doubly sublimed prior to use. Each of the substituted phenyl acetates used in this study was synthesized by following the general procedure of Pocker and

[1] Abbreviations used that are not listed in Biochemistry 5, 1445 (1966), are: CA, carbonic anhydrase; BCA, bovine carbonic anhydrase; HCA, human carbonic anhydrase; BCA A and BCA B denote the isozymes A and B of BCA; HCA B and HCA C denote the isozymes B and C of HCA.

[2] Submitted for publication.

TABLE I: Physical Properties of Substituted Phenols and Substituted Phenyl Acetate Esters.[a]

Phenyl Substituent	Phenol, Mp (C°)	Acetate Ester, Mp (C°)	Acetate Ester, Bp (0.1 mm) (°C)	Acetate Ester Refractive Index (n^{20}D, deg)
Unsubstituted	35.5–36		30.8–31.2	1.5037
p-Methyl	28–29		37.5–38.2	1.5026
p-Ethyl	38.5–39		49.0–50.0	1.5039
p-Isopropyl	59.5–60		54.8–55.2	1.4960
p-tert-Butyl	101–102		74.8–75.2	1.4989
p-Methoxy	52.5–53		54.0–55.0	1.5138
p-Chloro	38.5–39		41.5–42.5	1.5197
p-Carboxy	219–220	199.5–200		
p-Phenyl	166.5–167	79–80		

[a] All of the phenols were white solids and the esters were either clear liquids or white solids.

Storm (1968) for the preparation of p-nitrophenyl esters (see also, Spasov, 1938; Huggins and Lapides, 1947). The respective phenol (0.2 mole) was refluxed for 3 hr with freshly distilled acetyl chloride (0.24 mole) in the presence of powdered magnesium metal (0.20 g-atom) in 200 ml of benzene. Esterification time for all of the substituted phenols used was under 3 hr by which time evolution of HCl had ceased. Traces of phenol, HCl, and acetyl chloride were removed by repeated washings with saturated aqueous sodium bicarbonate followed by distilled water. The benzene layer containing the ester was dried over anhydrous sodium bisulfate and the benzene removed by rotary evaporation at reduced pressure. The resulting liquid esters were purified by fractional vacuum distillation. The solid esters were recrystallized from dioxane and then vacuum sublimed. Structural identification was confirmed from ir and nuclear magnetic resonance (nmr) spectra. In addition, a weighed amount of each ester was hydrolyzed and found to give quantitative release of the respective phenol as determined by uv absorption. The physical properties of the phenols and phenyl acetate esters appear in Table I.

Bovine carbonic anhydrase was prepared from commercial samples obtained from Mann Research Laboratories as described earlier (Pocker et al., 1971). Concentrations based on 1.00 ± 0.05 atom of zinc per molecule (determined by atomic absorption spectrophotometry), assuming a molecular weight of 30,000, were in accord with a molar extinction coefficient, ϵ_M, at 280 nm of 54,000 M^{-1} cm^{-1}. The electrophoretic pattern obtained in 7% polyacrylamide gels on a Canalco Model 12 EPH apparatus and chromatography on DEAE-cellulose revealed that the BCA was a mixture of A and B isomers in the ratio ca. 1:2. While the two isozymes differ in electrophoretic mobility, they have essentially identical amino acid composition and catalytic properties (Nyman and Lindskog, 1964; Pocker and Stone, 1967; Pocker and Dickerson, 1968). The BCA showed no loss of activity when stored at −20° under anhydrous conditions. Although aqueous stock solutions of the enzyme appeared to be quite stable at 25°, freshly prepared solutions were always used.

Buffer Components and Solutions. The buffers employed in this study are listed in Table II. Tris was obtained from Aldrich and purified by sublimation. Triethylamine was obtained from Eastman Kodak Co. and purified by fractional distillation, bp 89–90° (760 mm). Malonic acid (Aldrich) was recrystallized from ethanol–ether, mp 135°. Diethylmalonic acid was pre-

pared by hydrolysis of the diethyl ester (Aldrich) with alcoholic KOH and recrystallization of the acid from ether–benzene, mp 128–129°. Reagent grade monobasic sodium phosphate, dibasic sodium phosphate, sodium chloride, sodium hydroxide, and acetic acid were obtained from Allied Chemical and used without further purification. The total buffer concentration used in this work was maintained constant at 0.05 M throughout the pH range studied. The ionic strength was maintained constant at 0.09 by adding an appropriate amount of NaCl.

Acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, was obtained from American Cyanamide Co. (Lederle Laboratory Division) and ethoxzolamide, 6-ethoxy-2-benzothiazolesulfonamide, from the Upjohn Co. Reagent grade acetonitrile (Baker Analyzed) was used as a solvent for preparation of stock solutions of the various esters.

Instrumentation. The initial work on rates of substituted phenyl acetate ester hydrolysis was performed using a Beckman DU-2 spectrophotometer equipped with an insulated cell compartment consisting of a specially constructed bath thermostatted to 25.0 ± 0.02° by means of a Sargent Model SV (S-82060) thermonitor. The instrument has recently been re-

TABLE II: Buffer Systems Employed for Kinetic Studies.

Buffer[a]	Step	pK_a (25.0°)[b]	pH Range Used
Acetate		4.71	4–5
Phosphate	2	7.21	6–7.5
Malonate	1	2.85	2–4.5
	2	5.66	4.5–6.5
Diethylmalonate	1	2.20	2–3
	2	7.23	6.2–8.2
Tris		8.08	7.5–9.3
Triethylamine acetate		10.67	9.5–11.4

[a] Only sodium salts were used in buffer preparation and NaCl was added as necessary to maintain constant ionic strength (with triethylamine, sodium acetate was used in place of sodium chloride). [b] The various pK_a values were taken from either the Handbook of Chemistry and Physics (Weast, 1968) or Perrin (1964).

TABLE III: Spectral Data.

Compound	Buffer[a] pH	λ_{max} (nm)	λ_{iso}[b] (nm)	log ϵ	Compound	Buffer[a] pH	λ_{max} (nm)	λ_{iso}[b] (nm)	log ϵ
Phenol	2	269.5		3.18	p-tert-Butylphenol	2	274		3.18
	11.5	235		3.95		11.5	237		4.02
	11.5	287.5		3.37		11.5	292.5		3.35
	2–12		275	3.12		2–12		278	3.12
Phenyl acetate	7	257.5		2.39	p-tert-Butylphenyl	7	269		2.62
	7	264		2.35	acetate	7	262		2.59
	7		275	1.89		7		278	1.99
p-Methylphenol	2	277.5		3.23	p-Methoxyphenol	2	287.5		3.40
	11.5	237.5		3.88		11.5	235		3.92
	11.5	296.5		3.37		11.5	305.5		3.34
	2–12		281.8	3.17		2–12		295	3.31
p-Methylphenyl acetate	7	264		2.70	p-Methoxyphenyl acetate	7	264		2.70
	7	271		2.69		7	271		2.69
	7		281.8	1.98		7		295	1.85
p-Ethylphenol	2	276.5		3.23	p-Chlorophenol	2	280		3.18
	11.5	237.5		3.92		11.5	244		4.04
	11.5	294		3.35		11.5	298		3.36
	2–12		282	3.14		2–12		283.5	3.12
p-Ethylphenyl acetate	7	270		2.78	p-Chlorophenyl acetate	7	266.5		2.59
	7	264		2.74		7	273.5		2.52
	7		282	2.42		7		283.5	1.52
p-Isopropylphenol	2	276.5		3.00	p-Carboxyphenol	2	255		4.16
	11.5	237.5		3.98		11.5	280		4.23
	11.5	294		3.37		2–12		263	4.08
	2–12		280	3.16	p-Carboxyphenyl acetate	7	262.5		4.02
p-Isopropylphenyl acetate	7	262.5		2.65		7		263	3.00
	7	268.5		2.61	p-Phenylphenol	2	257.5		4.25
	7		280	1.48		11.5	287.5		4.28
						2–12		271.5	4.16
					p-Phenylphenyl acetate	7	251.5		4.28
						7		271.5	3.90

[a] In each case, the solvent was a buffered aqueous solution containing 1% or 10% (v/v) acetonitrile. [b] λ_{iso} was the wavelength where $\epsilon_{phenol} = \epsilon_{phenolate\ anion}$. The ϵ of each phenyl acetate is also given at the λ_{iso} for the respective phenol.

Placed with a Beckman Kintrac VII automated spectrophotometric system. The temperature is controlled at 25.0° ± 0.02° by a Forma-Temp Jr. (Model 2095) circulating bath attachment. All pH determinations were made with a Beckman 101900 research pH meter equipped with a Beckman calomel internal 39071 frit junction reference electrode and a Beckman glass electrode 41263. Nuclear magnetic resonance spectra for structure verification were obtained with a Varian Associates A-60 instrument. Uv spectra were obtained on a Cary 14 recording spectrophotometer. Ir spectra were obtained on a Perkin-Elmer 137 sodium chloride spectrophotometer. The determination of zinc content in BCA was done on a Beckman atomic absorption accessory (130000) attached to a Beckman DU-2 spectrophotometer.

Kinetic runs for determination of buffer rates were performed in a 10-mm rectangular silica cell. A solution (0.30 ml) of acetonitrile containing substrate was injected by means of a calibrated Yale syringe into 2.7 ml of the appropriate buffer giving a final solution of acetonitrile 10% (v/v) and $\mu = 0.09$. The acetonitrile stock solutions of the esters could be prepared and stored for long periods of time with no decomposition. While the acetonitrile served to increase the

solubility of the esters and was transparent at wavelengths above 270 nm, it had the disadvantage of slightly inhibiting BCA activity (Pocker and Stone, 1965, 1967).

The hydrolysis of each substituted phenyl acetate was followed spectrophotometrically by monitoring the appearance of phenol or phenolate anion at the isosbestic point (where $\epsilon_{phenol} = \epsilon_{phenolate\ anion}$, Table III). Each substituted phenyl acetate had a small contributing absorption at that point (Table III) and this was corrected for in calculating the rates. The p-phenylphenyl acetate, however, had a high absorption at 271.5 nm (the isosbestic point for p-phenylphenol) so it was necessary to follow the hydrolysis of this acetate at 288 nm (a λ_{max} for p-phenylphenolate anion) where the ester does not absorb appreciably.

The hydrolysis of each substituted phenyl acetate ester proceeded to > 99% completion. Pseudo-first-order rate constants were determined by plotting $-\log (A_\infty - A_t)$ vs. time. Values of A_∞ were experimentally determined after at least ten half-lives and were found to reach a stable value. In the determination of the pH–rate profiles, a concentration sufficient to give $A_\infty \sim 1$ was chosen for each substrate while the enzyme concentration was held at 10^{-4} M. At these substrate concentra-

TABLE IV: Rate Constants for the Hydrolysis of Substituted Phenyl Acetate Esters.

Acetate	k_w [a] $(\min^{-1} \times 10^4)$	Lit. k_w $(\min^{-1} \times 10^4)$	Lit. k_{Im} $(M^{-1}\ \min^{-1})$	k_{OH^-} [a] $(M^{-1}\ \min^{-1})$	Lit. k_{OH^-} $(M^{-1}\ \min^{-1})$
Phenyl	<0.5	<20[b] 1.35[c]	0.52[b]	75	76[b]
p-Methylphenyl	<0.5	<10[b] 1.73[c]	0.21[b]	70	59[b]
p-Ethylphenyl	<0.5			65	
p-Isopropylphenyl	<0.5			65	
p-Methoxyphenyl	<0.5	<10[b]	0.19[b]	70	63[b]
p-Chlorophenyl	<0.5	<100[b]	1.6[b]	130	132[b]
p-Carboxyphenyl	<0.5			90	
p-Phenylphenyl	<0.5			75	
p-Nitrophenyl		100[b] 43.7[c]	35[b] 21.3[c]	890	570[b] 890[d]

[a] Determined at 25.0°; $\mu = 0.09$, in 10% (v/v) acetonitrile (this work). [b] Determined at 25.0°; $\mu = 1.00$, up to 1% (v/v) acetonitrile (Kirsch and Jencks, 1964). [c] Determined at 30°; $\mu = 0.2$, in 28.5% (v/v) ethanol–water (Bruice and Schmir, 1957). [d] Determined at 25.0°; $\mu = 0.09$, in 10% (v/v) acetonitrile (Pocker and Stone, 1967).

tions, plots of $-\log (A_\infty - A_t)$ vs. time were linear for at least two half-lives.

For the determination of enzymatically catalyzed rates of hydrolysis, high enzyme concentrations (10^{-4} M) were required. Because BCA absorbs (ϵ_M 54,000 M^{-1} cm^{-1} at 280 nm) in the region of the isosbestic point of the substituted phenols (λ_{iso} from 275–295 nm, Table III), a solution of the enzyme equal in concentration to that employed in the reaction cell was used in the reference cell. In addition, 2- and 5-mm cells were used in order to reduce total absorption due to enzyme. With these narrower cells, reproducibility was greatly improved because it became possible to balance the instrument using slit widths as narrow as 0.6 mm.

Equation 1 gives the expression for the buffer rates,

$$k_{buff} = k_0 + k_{H_3O^+}[H_3O^+] +$$
$$k_{OH^-}[OH^-] + k_{HB^+}[HB^+] + k_B[B] \quad (1)$$

where k_0 is the catalytic constant for the water-catalyzed reaction, and the various k's are catalytic constants for each species present in the system. HB$^+$ and B are the acidic and basic forms of the buffer. The enzyme component was determined from the rate with the buffered enzyme solution using eq 2

$$k_{enz} = [k_{obsd} - k_{buff}](1/[E]) \quad (2)$$

One of the major difficulties with most of the substituted phenyl acetates was that the enzyme component $k_{enz}[E]$ was not much larger than that associated with the value of $k_{OH^-}\cdot$ [OH$^-$] above pH 10. This fact precluded the accurate determination of pH–rate profiles at high pH. In the Michaelis constant determination, high substrate concentrations were encountered so that the total absorbance sometimes exceeded 3.0. When this occurred theoretical A values were used since the spectrophotometer response was no longer linear. In addition, at high substrate levels, initial rates were calculated since pH changes during hydrolysis sometimes exceeded 0.01 pH units.

Pseudo-first-order rate constants for substituted phenyl

acetate ester hydrolysis were evaluated using a Fortran IV computer program executed on a CDC 6400 digital computer. The program was written by Dr. Nori Watamori to calculate the test slope for first-order plots by means of the least-squares method. It gives the rate constant, the reaction half-life, the standard deviation of the slope, and the correlation coefficient. In addition, when the correlation coefficient is less than 0.999, the data are graphically plotted so that the operator can quickly pinpoint the cause of the discrepancy.

Inhibition Studies. Per cent inhibition of carbonic anhydrase activity by acetazolamide and ethoxzolamide was determined as a function of added inhibitor for each of the phenyl ace-
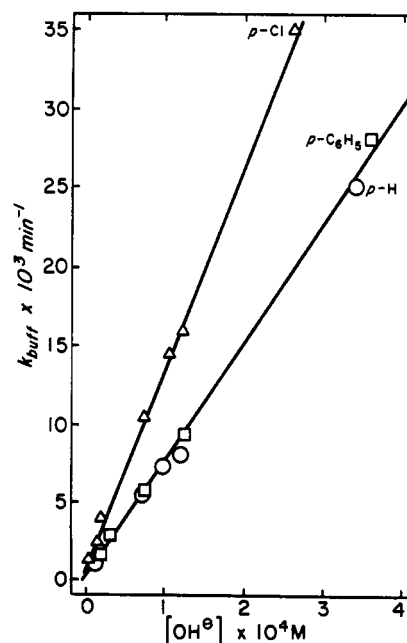

FIGURE 1: Plots of k_{buff} vs. [OH$^-$] for the hydrolysis of phenyl acetate esters at 25.0°; 10% (v/v) acetonitrile; ionic strength, $\mu = 0.09$. O, phenyl acetate; □, p-phenylphenyl acetate; △, p-chlorophenyl acetate

TABLE V: pH Dependency of k_{enz} for the Hydrolysis of Substituted Phenyl Acetate Esters at 25.0°.[a]

Acetate Ester	pH	Buffer	k_{enz}	Acetate Ester	pH	Buffer	k_{enz}
Phenyl	6.02	P	6	p-Methoxyphenyl	6.2	P	8
	6.92	P	50		7.28	P	50
	7.42	P	84		8.25	T	78
	8.31	T	185		8.74	T	114
	9.08	T	196		9.08	T	129
	10.11	TA	196		9.70	TA	126
	10.53	TA	196	p-Chlorophenyl	6.16	P	64
p-Methylphenyl	6.01	P	4		6.69	P	240
	7.00	P	56		7.28	P	615
	7.30	P	91		7.35	P	670
	8.04	T	160		7.93	T	1150
	8.97	T	189		8.44	T	1440
	10.04	TA	189		8.90	T	1440
	10.22	TA	190		9.09	T	1540
	10.35	TA	195		9.87	TA	1440
p-Ethylphenyl	6.20	P	0		10.05	TA	1470
	6.60	P	32		10.12	TA	1470
	7.21	P	105	p-Carboxyphenyl	6.6	P	11
	8.26	T	167		7.47	P	42
	8.84	T	189		8.00	T	56
	9.83	TA	217		9.14	T	55
	10.44	TA	217		10.09	TA	70
p-Isopropylphenyl	6.60	P	32		10.45	TA	73
	7.28	P	112	p-Phenylphenyl	6.15	P	200
	8.21	T	193		6.60	P	700
	8.86	T	224		7.36	P	1740
	9.86	TA	203		7.46	P	2150
	10.07	TA	224		8.23	T	3380
	10.41	TA	252		8.50	T	4060
					9.28	T	4200
					9.54	TA	4200
					9.60	TA	4100
					10.14	TA	4200

[a] Values of k_{enz} are determined in 0.05 M buffer, ionic strength, μ = 0.09 with 10% (v/v) acetonitrile from the equation k_{enz} = $(k_{obsd} - k_{buff})/[E]$, where [E] is the enzyme concentration based on zinc content assuming a molecular weight of 30,000. Buffer abbreviations are: P (phosphate), T (Tris), and TA (triethylamine–acetate).

tates. Concentration of enzyme was 5 × 10⁻⁵ M; acetazolamide or ethoxzolamide concentrations were varied from 5 × 10⁻⁶ to 5 × 10⁻⁵ M. Substrate concentrations were selected so that A_∞ was near unity.

Results

For the substituted phenyl acetate esters, the buffer hydrolysis curve is almost exclusively dictated by hydroxide ion catalysis. This can be seen in Figure 1, which shows representative plots of k_{buff} vs. [OH⁻] for phenyl acetate, p-chlorophenyl acetate, and p-phenylphenyl acetate. From eq 1, it can be seen that the intercept, I in Figure 1, can be expressed by eq 3

$$I \simeq 0 = k_0 + k_{H_3O^+}[H_3O^+] + k_{HB^+}[HB^+] + k_B[B] \quad (3)$$

Since the rate constants of eq 3 were all small compared to k_{OH^-}, no attempt was made to evaluate them. The k_{OH^-} values as determined from the slope of plots of k_{buff} vs. [OH⁻] appear in Table IV.

The value of k_{enz} as a function of pH was detemined for each ester (Table V) as described in the Experimental Section. The pH–rate profiles appear in Figure 2; for all of the esters the profile is sigmoidal with an inflection near pH 7.4. The similarities between these profiles and those observed in the carbonic anhydrase catalyzed hydrations of CO₂ (Kernohan, 1965; Lindskog, 1966), aldehydes (Pocker and Meany, 1965a,b), and hydrolysis of p-nitrophenyl acetate (Pocker and Stone, 1965, 1967; Thorslund and Lindskog, 1967; Verpoorte et al., 1967) suggest that the same general mechanism is operating in all these reactions. For a comparison of the para substituent effects, the plateau value of k_{enz} for each phenyl acetate ester is given in Table VI. In addition the corresponding value of the ratio, k_{enz}/k_{OH^-}, is also included. The fact that this ratio varies significantly from one ester to another indicates that the magnitude k_{enz} is not dictated merely by the reactivity of the ester toward nucleophilic attack.

With these esters, no initial "burst" of release of the respective phenol was observed indicating that during ester hydrolysis an acetyl-enzyme intermediate either does not form or hydrolyzes very rapidly. Consequently, the dependence of

TABLE VI: Substituent Constants and Second-Order Rate Constants for Substituted Phenyl Acetate Ester Hydrolysis at 25.0°.

Acetate Ester	π^a	σ^{-b}	k_{OH^-} (M^{-1} min^{-1})	$k_{enz}{}^c$ (M^{-1} min^{-1})	k_{enz}/k_{OH^-}
Phenyl	0	0	75	195	2.6
p-Methylphenyl	0.50	−0.17	70	195	2.8
p-Ethylphenyl	1.00	−0.15	65	217	3.3
p-Isopropylphenyl	1.30	−0.15	55	224	4.1
p-Phenylphenyl	2.00	0.009	75	4200	56
p-Methoxyphenyl	−0.12	−0.27	70	125	1.8
p-Carboxyphenyl	−0.32^d	0.27^d		e	
	−4.36^f	0.13^f	90	70^e	0.8
p-Nitrophenyl	0.28	1.24	890	24000	27
p-Chlorophenyl	0.70	0.23	130	1470	11

^{a} From Hansch (Fujita *et al.*, 1964; Hansch *et al.*, 1965) assuming that the π values for phenyl acetate esters are the same as the π values for substituted benzenes. ^{b} From Jaffe (1953). ^{c} Determined at pH 8.90, see Table V. The value k_{enz} for pNPA is in agreement with the value of 2.4 × 10^4 M^{-1} min^{-1} at pH 8.92 determined by Pocker and Stone (1965, 1967). ^{d} Values for the carboxylic acid. ^{e} The correct values of π and σ^- for p-carboxyphenyl acetate are not known. Thus, in the enzyme cavity this compound may exist as the carboxylate anion, as the hydrogen-bonded anion, as the tight or solvent-separated zinc–carboxylate ion pair, or even as the carboxylic acid. Consequently, the rate data for p-carboxyphenyl acetate was not used in the computerized regression analysis. ^{f} Values for the carboxylate anion.

enzymatic rate on substrate concentration was formally analyzed in terms of the simplified Michaelis–Menten (1913) scheme (eq 4)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} E + P \tag{4}$$
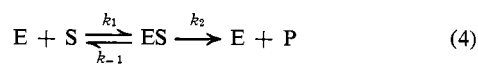
It is of course realized that the specific rate constants determined experimentally may be considerably more complex than those indicated by this simplified scheme. Since k_{enz} reflects the combined ability of an enzyme to bind and hydrolyze a substrate, it is valuable to use Lineweaver–Burk (1934) plots to determine formal values of $K_m = (k_{-1} + k_2)/k_1$ and $V_m = k_2[E]$. Using this procedure, substituent effects on substrate binding should clearly be separable from substituent effects on the magnitude of the turnover, k_2. Unfortunately, as a result of the limited substrate solubility, $K_m \gg [S]$, even in 10% (v/v) acetonitrile, the experimental data were limited to the linear portion of v *vs.* [S] and hence plots of $1/v$ *vs.* 1/[S] were found to go through the origin. Figure 3 shows a representative Lineweaver–Burk plot for p-chlorophenyl acetate.

For all of the substituted phenyl acetate esters, the sulfonamides, acetazolamide, and ethoxzolamide, proved to be potent inhibitors of esterase activity. Figure 4 shows a representative plot of per cent enzymatic activity *vs.* added ethoxzolamide or acetazolamide for the hydrolysis of phenyl acetate. The results were the same for all esters studied (Table VII). Plots of per cent enzymatic activity *vs.* the ratio of sulfona-
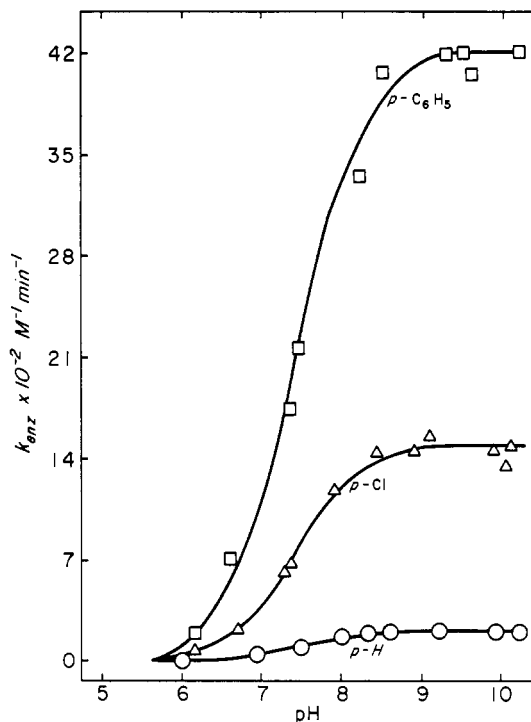

FIGURE 2: pH–rate profiles for the BCA-catalyzed ester hydrolysis at 25.0°; 10% (v/v) acetonitrile; μ = 0.09. O, phenyl acetate; △, p-chlorophenyl acetate; □, p-phenylphenyl acetate.

mide inhibitor to enzyme concentration all show 1:1 loss of activity with added inhibitor. When the inhibitor concentration was equal to the total enzyme concentration, inhibition was >98% complete. This indicates that there is only one esteratic site per enzyme molecule.

Both acetazolamide and ethoxzolamide were assumed to be noncompetitive inhibitors of substituted phenyl acetate hydrolysis. Since K_m could not be determined for these substrates, it proved difficult to test this point directly. However, it seems reasonable that the inhibition of the hydrolysis of
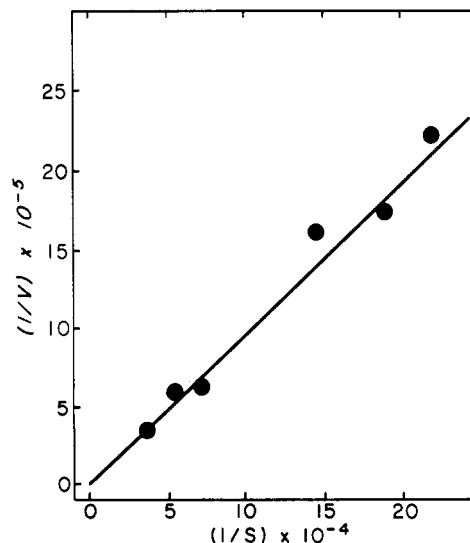

FIGURE 3: Lineweaver–Burk plot for p-chlorophenyl acetate hydrolysis at 25.0°; 10^{-4} BCA in 0.05 M Tris and 10% (v/v) acetonitrile, pH 8.7, μ = 0.09
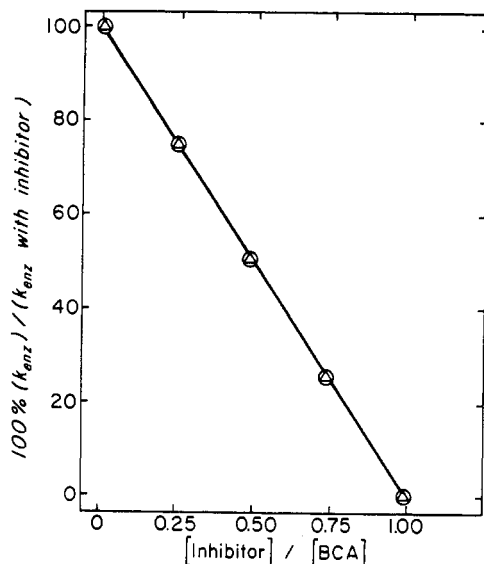
FIGURE 4: Representative plot of BCA activity as a function of added inhibitor for enzyme-catalyzed hydrolysis of phenyl acetate at $25.0°$; 5×10^{-5} M BCA in 0.05 M Tris buffer and 10% (v/v) acetonitrile, pH 8.9, $\mu = 0.09$. O, ethoxzolamide; $\triangle$, acetazolamide.

TABLE VII: Effect of Inhibitors on the BCA-Catalyzed Hydrolysis of Substituted Phenyl Acetate Esters at $25.0°$.

Acetate Ester	Inhibitor[a] I	[I] ($\times$ 10^5 M)	k_{enz}[b]	[I]/[E]	% Enzymatic Act.[c]
Phenyl	A or E	0	140	0	100
	A	1.25	101	0.25	72
	A	2.50	70	0.50	50
	A	3.75	36	0.75	26
	A	5.00	3	1.00	2
	E	1.25	103	0.25	74
	E	2.50	69	0.50	50
	E	3.75	35	0.75	25
	E	5.00	0	1.00	0
p-Methylphenyl	A or E	0	140	0	100
	A	2.50	73	0.50	49
	E	2.50	70	0.50	50
p-Ethylphenyl	A or E	0	155	0	100
	A	2.50	78	0.50	50
p-Isopropylphenyl	A or E	0	160	0	100
	A	2.50	80	0.50	50
p-Methoxyphenyl	A or E	0	90	0	100
	A	2.50	44	0.50	49
p-Chlorophenyl	A or E	0	1050	0	100
	A	1.25	770	0.25	73
	A	2.50	525	0.50	50
	A	3.75	265	0.75	25
	A	5.00	0	1.00	0
	E	1.25	775	0.25	75
	E	2.50	525	0.50	50
	E	3.75	264	0.75	25
	E	5.00	0	1.00	0
p-Carboxyphenyl	A or E	0	50	0	100
	A	2.50	25	0.50	50
p-Phenylphenyl	A or E	0	3000	0	100
	A	1.25	2250	0.25	75
	A	2.50	1500	0.50	50
	A	3.75	750	0.75	25
	A	5.00	15	1.00	0.5
	E	1.25	2200	0.25	73
	E	2.50	1500	0.50	50
	E	3.75	750	0.75	25
	E	5.00	0	1.00	0

[a] The abbreviations are: A (acetazolamide) and E (ethoxzolamide). [b] $k_{enz} = (k_{obsd} - k_{buff})/[E]$. The BCA concentration was held constant at 10^{-4} M in 0.05 M Tris buffer, $\mu = 0.09$, pH 8.9. The value of k_{obsd} decreases with increasing inhibitor concentrations but the value of k_{buff} is not affected by either of the inhibitors. [c] The per cent activity is [(k_{enz} with inhibitor)/ (k_{enz} without inhibitor)] $\times$ 100%.

these substrates would parallel that found for p-nitrophenyl esters, aldehydes, and CO_2. Similarly, values were not determined for the inhibition constant K_i, the dissociation constant for the enzyme–inhibitor complex. With 10^{-4} M BCA, both acetazolamide and ethoxzolamide are so powerfully bound to the enzyme that even with inhibitor concentrations approaching 10^{-4} M, virtually all of the inhibitor is bound to the enzyme. From work with p-nitrophenyl acetate, the constant K_i can be estimated to be around 2×10^{-7} M (Pocker and Stone, 1968a). Even in this case, the number ascribed to the inhibition constant is only an apparent value as the concentration of free inhibitor will be minute, therefore making it difficult to evaluate a true dissociation constant (Webb, 1963).

Discussion

The substrates chosen for this correlative study were substituted phenyl acetate esters. The advantage in using these esters is that a great deal is known about the effects of para substituents on their rate of chemical hydrolysis (Bruice and Schmir, 1957; Bruice and Mayahi, 1960; Kirsch and Jencks, 1964; Amaral et al., 1967), and it was hoped that more light would be shed on the nature of the esterase activity of erythrocyte CA.

However, with less reactive phenyl acetates, the highest attainable $[S]_0$ values were below the respective K_m values and consequently it will be necessary to proceed with the discussion in a more qualitative fashion. There is but one esteratic site per enzyme molecule (this work; Pocker and Storm, 1968; Pocker and Stone, 1968a,b) which is located in a deep crevice with the chelated zinc ion situated at the bottom of the cleft (Fridborg et al., 1967). The zinc ion is essential for both hydrase and esterase activity (Malmstrom et al., 1964; Pocker and Meany, 1964, 1965a,b, 1967a,b; Duff and Coleman, 1966; Pocker and Stone, 1967). We confirm here the earlier finding that removal of zinc abolishes the esterase activity. A small degree of reactivation (ca. 0.1 of that of the native enzyme) can be achieved with Mn^{2+} but only Zn^{2+} and Co^{2+} are really efficient reactivators. The metal ion specificity of the enzyme, in the

region $[S_0] \ll K_m$, is essentially the same for both CO_2 hydration and pNPA hydrolysis ($k_{enz}^{Zn^{2+}}/k_{enz}^{Co^{2+}} \approx 1$) although the values of k_{enz} differ by a factor of 10^5 for the two reactions (Thorslund and Lindskog, 1967; Y. Pocker and J. T. Stone, unpublished observations). Thus, it appears that in contrast to carboxypeptidase A (Coleman and Vallee, 1961), the metal ion specificity of BCA is nearly independent of the nature of the substrate.

The esterase activity of erythrocyte CA is noncompetitively inhibited by monovalent anions. The binding of one such anion per enzyme molecule suffices to abolish its catalytic activity (Lindskog, 1963, 1966; Pocker and Stone, 1965, 1967, 1968a). The inhibitor appears to be bound close to the metal ion, pointing from the zinc ion into the deeper part of the crevice, at a distance no greater than that required for the formation of a solvent-separated ion pair (Fridborg et al., 1967; A. Liljas, private communication, 1970). Hence it is generally assumed that the neutral esters are bound in a hydrophobic region slightly removed from the zinc ion, but the steric and electronic requirements of this binding are as yet only partially understood. Actually, from the fact that the k_{enz}/k_{OH}-ratio (see Table VI) differ widely between the various para-substituted phenyl acetate esters, it is evident that in addition to the relative chemical reactivity of the substrate, other factors affected the rate of enzymatic hydrolysis. Indeed, it was noted earlier that subtle differences in substrate structure lead to significant differences in binding (Koshland, 1956, 1962; Pocker and Meany, 1967a,b; Pocker and Dickerson, 1968; Y. Pocker and L. J. Guilbert,[2] 1972).

Another aspect of the present work pertains to a consideration of the possible change in the rate-determining step with the changing substrate structure. It is reasonable to assume that the reaction of hydroxide ion with a series of para-substituted phenyl esters proceeds by a mechanism, in which the attack by OH^- is largely or entirely rate controlling so that k_{OH}- is a measure of the reactivity of the ester toward nucleophilic attack. On the other hand, with weaker nucleophiles when the leaving group is made sufficiently poor, the tetrahedral intermediate, if it is formed, will break down to expel the attacking rather than the leaving group and will then give back starting materials instead of products, resulting in a break in the structure–reactivity correlation. Since the basic form of a group with a pK near 7 is required for activity we were interested to find out if this group behaves as nucleophile or as a general base. Thus if the pK_a for the ionization of the zinc–aquo complex in the native enzyme were around 7 (first inflection in the pH–rate profile) then the nucleophilicity of its conjugate zinc-hydroxo complex would be such that with p-nitrophenylacetate the rate-determining step would involve attack of the preformed zinc-hydroxo complex on the ester whereas with phenyl acetate and the less reactive acetates the rate-controlling step would be expected to be the breakdown of the tetrahedral intermediate. On the other hand, if the true nucleophilicity of the zinc-hydroxo complex were to be associated with a group of pK_a around 11 (Pocker and Storm, 1968; Pocker and Stone, 1968b) and the basic group of pK_a near 7 acted merely as a general base, then the rate-determining step would remain invariant in the series of substituted phenyl acetates used in this work; a conclusion which indeed applies to the present case.[3]

In problems of mechanism, illumination often follows from a detailed analysis of specific inhibitors. Therefore considerable interest attaches to the binding characteristics of benzenesulfonamides. The sulfonamide binding site consists of at least two regions, one a hydrophobic region which attracts the aromatic portion of the sulfonamide (Chen and Kernohan, 1967; Galley and Stryer, 1968; Lindskog, 1969), the other a zinc ion containing region which is required for the strong binding of the inhibitor (Lindskog, 1963; Coleman, 1967). Lindskog (1969) has attempted to correlate inhibitor binding strength with Hammett's σ but found that no such correlation exists. Independently, Kakeya et al. (1969a) determined the binding strengths of 22 sulfonamide inhibitors of carbonic anhydrase, and correlated them (Kakeya et al., 1969b) using a linear combination of Hammett's σ and hydrophobic π (Fujita et al., 1964)[4] parameters. Taylor et al. (1970) have directly determined the kinetics of reaction between human carbonic anhydrases B and C and aromatic sulfonamides. They postulate that conformational changes induced in the enzyme (Koshland, 1956, 1962; Koshland and Neet, 1968) during binding (Taylor et al., 1970; King and Burgen, 1970; King and Roberts, 1971) affect the strength of binding. However, they give no method for predicting the thermodynamic stability of the enzyme–sulfonamide complexes.

Phenyl esters are bound in a hydrophobic region slightly removed from the sulfonamide region (Pocker and Stone, 1965, 1968a; Verpoorte et al., 1967; Thorslund and Lindskog, 1967; Pocker and Storm, 1968), but it is generally assumed that factors favoring the binding of sulfonamides parallel those for phenyl acetate esters. It appears therefore, that it would be informative to correlate the $k_{enz} \cong k_2/K_m$ values for substituted phenyl acetates with the free-energy terms π and σ. Dr. Corwin Hansch was kind enough to help us evaluate the separate electronic and adsorption effects on the rates of our enyzmatic reactions. The constants in eq 5 and 6 were obtained using a computerized regression analysis,

$$\log k_{enz} = 2.66 + 1.47\sigma^- \qquad \begin{array}{ccc} n & r\cdot & s \\ 8 & 0.870 & 0.442 \end{array} \qquad (5)$$

$$\log k_{enz} = 2.31 + 0.49\pi + 1.80\sigma^- \qquad 8 \quad 0.960 \quad 0.273 \qquad (6)$$

where n is the number of substrates, r the correlation coefficient, and s the standard deviation. Equation 6 is a statistically significant improvement over eq 5 ($F_{1,5} = 20.7$; $F_{1,5\alpha.01} = 16.3$; for a discussion of the F test, see Draper and Smith, 1966), and illustrates the significance of the hydrophobic

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[3] The present data are best interpreted in terms of an esterase activity actuated by a group acting as a general base rather than as a nucleophile. Of course, kinetic data per se often characterizes but seldom identifies a group at the active center. The base in question is derived from a conjugate acid which in its special enzymatic environment has a pK_a value near 7. Our working hypothesis is that this group is a histidine residue hydrogen bonded through water bridges to the zinc-aquo complex but it has not escaped our attention that the zinc-hydroxo complex by itself may act as a general base rather than as a nucleophile as is often assumed. We defer comment on the intrinsic pK_a of the zinc-aquo complex in bovine carbonic anhydrase B and wish only to point out that our modification studies (Pocker, 1969; Y. Pocker and J. T. Stone, unpublished observations) have clearly identified a specific histidine residue at or near the active site. We have furthermore shown

that the enhanced activity at high pH, while independent of the specific histidine residue, is nevertheless associated with parts of the same active center. Indeed, the catalytic activity of bovine carbonic anhydrase B is not significantly affected by the modification of all lysine and arginine residues and of the one exposed tyrosine residue. On the other hand, the high pH esterase activity is completely eradicated by the removal of zinc. Here one may note that the enhanced activity at high pH is associated both with significant conformational changes and with the ionization of a group of pK_a = >10.

[4] In order to explain the large binding energies which are found in enzyme–substrate interactions, it is frequently necessary to invoke hydrophobic forces. A binding parameter π, which is a measure of possible hydrophobic binding energy of a given compound comparable to the σ parameter for polar effects (Jaffe, 1953), has been defined by Hansch (Fujita et al., 1964) and is based on partition coefficients between water and octanol. The slope of a logarithmic plot of binding constants against this parameter for a series of compounds is a measure of the extent to which the parameter in the binding process resembles transfer from water to octanol.

TABLE VIII: Difference between Observed and Predicted Values of k_{enz}.

Acetate Ester	Obsd Log k_{enz}	Predicted[a] Log k_{enz}	Deviation
Phenyl	2.26	2.31	−0.05
p-Methylphenyl	2.26	2.25	0.01
p-Ethylphenyl	2.30	2.53	−0.23
p-Isopropylphenyl	2.32	2.68	−0.36
p-Carboxyphenyl	1.81	b	b
p-Methoxyphenyl	2.08	1.82	0.26
p-Chlorophenyl	3.13	3.07	0.06
p-Phenylphenyl	3.59	3.26	0.33
p-Nitrophenyl	4.38	4.40	−0.02

[a] Predicted from the equation log k_{enz} = 2.31 + 0.49π + 1.80σ^-, using π and σ^- values from Table VI. [b] It should be noted that the appropriate π and σ^- values are not known in this case (Table VI, footnote e). It is attractive to assume that the negative charge on the carboxyl function may facilitate its attachment to the anionic binding site situated near the zinc ion in the native enzyme. Therefore, it is not surprising that this compound does not fit the correlation represented by eq 6.

term. Moreover, the coefficient associated with this term is close to that normally found for such processes (Helmer et al., 1968). A listing of observed values of log k_{enz} vs. those predicted by eq 6 appears in Table VIII.

In conclusion, it is seen that the application of regression analysis using the free-energy-related terms π and σ provides a reasonable explanation for structure–reactivity relations for substituted phenyl acetate esters. Using eq 6 it should be possible to roughly predict the value of k_{enz} for many substituted phenyl acetate esters (except ortho-substituted compounds where steric hindrance effects would have to be considered).

Acknowledgment

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Influence of Substrates and Effectors on the Binding of 1-Anilino-8-naphthalenesulfonate by Glycogen Phosphorylase*

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ABSTRACT: The binding of 1-anilino-8-naphthalenesulfonate (ANS) to skeletal muscle glycogen phosphorylases a and b has been studied using a fluorescence method. Phosphorylase a, a tetramer, binds ANS more tightly than phosphorylase b, a dimer. The titration behavior of both forms of the enzyme at pH 7.1–7.2 under two conditions of ionic strength (0.03 and 0.23 M) indicates the presence of multiple binding sites with overlapping affinity. At low ionic strength (0.03 M), the average dissociation constant for the binding of less than 1 mole of ANS/protomer is approximately 10 μM for phosphorylase a and 50–70 μM for phosphorylase b. Increasing the ionic strength of the buffer to 0.23 M reduces the affinity of both phosphorylases a and b for ANS by about tenfold. Substrates and modifiers of enzymatic activity decrease the fluorescence of solutions containing ANS and phosphorylase b by as much as 85%. For the phosphorylase b–ANS complex, AMP, an activator, and glucose 6-phosphate, an inhibitor, are effective at the lowest concentrations followed by inorganic phosphate and glucose 1-phosphate. Less specific decreases in fluorescence are observed upon addition of salt; 0.4 M NaCl quenches the fluorescence of solutions of phosphorylase b and ANS by about 75%. Direct binding measurements were also made using equilibrium dialysis. A good correlation was found between the decrease in the fluorescence intensity and the decrease in the average number of ANS molecules bound either to phosphorylase a in the presence of AMP, or to phosphorylase b in the presence of AMP or glucose 6-phosphate. All of the effects observed are consistent with a noncompetitive model in which the binding of substrates or modifiers decreases the affinity of the sites which bind ANS.

R abbit muscle glycogen phosphorylase (EC 2.4.1.1) exists in two states of aggregation: phosphorylase b, a dimer inactive in the absence of AMP,[1] and phosphorylase a, a tetramer active in the absence of this nucleotide. The monomer has the following well recognized sites: an active site which binds substrates, a site which binds AMP, a specific seryl residue which can be phosphorylated in the b-to-a reaction, and a site which binds pyridoxal 5'-phosphate by weak covalent bonds. Binding of substrates increases the affinity of phosphorylases a and b for AMP and vice versa. References to the original literature can be found in the review by Cori (1969). Phosphorylase also binds several dyes including 1-anilino-8-naphthalenesulfonate (ANS) (Stryer, 1965), bromothymol blue (BTB) (Ullmann et al., 1964), and 2-methylanilinonaphthalene-6-sulfonate (MNS) (Birkett et al., 1970). This report describes the binding of ANS to phosphorylases a and b and the interaction which occurs between the ANS sites and the sites which bind substrates and modifiers of enzymatic activity.

[1] Abbreviations used are: AMP, adenosine 5'-monophosphate; ANS, 1-anilino-8-naphthalenesulfonate; BTB, bromothymol blue; glucose-1-P, glucose 1-phosphate; glucose-6-P, glucose 6-phosphate; K_d, dissociation constant; MNS, 2-methylanilino-6-sulfonate.