suggests that inorganic sulfate is most directly involved.

The curious temperature effect on the rate of enzyme synthesis is most probably not the result of heat inactivation of the enzyme. This possibility seems to be excluded on the basis of the optimum assay temperature and the temperature stability of the purified enzyme (Fowler and Rammler, 1964). The high (38–40°) and low (24°) temperature inhibition need not be directly related, but can represent two dissimilar events both of which effect enzyme synthesis. Although the enzyme shows no cofactor requirements (Fowler and Rammler, 1964), the possibility of a temperature-sensitive controlling element exclusive of the enzyme-synthesizing system may be entertained.

An enzymatic system analogous to the sulfatase is alkaline phosphatase of *E. coli* (Horiuchi *et al.*, 1959; Torriani, 1960). There are, however, some differences. Sulfatase synthesis is repressed by sulfate but its catalytic activity is not effected, while alkaline phosphatase synthesis and catalytic activity are inhibited by phosphate (Stadtman, 1961). When *A. aerogenes* is disrupted and the cellular debris is removed, all enzymatic activity can be recovered in the supernatant solution. Although we have been unable to effect lysozyme digestion of the bacterial cell wall under a variety of conditions, it would appear that the sulfatase, like alkaline phosphatase (Malamy and Horecker, 1961) is neither extracellular in nature nor tightly associated with particular cellular constituents. This system, like the acid phosphatase of *Saccharomyces cerevisiae* (McLellan and Lampen, 1963), appears to be another of a class of enzymes found on the periphery of the cell. Although its exact biological role is unknown it most probably functions in this soil bacterium as an arylsulfatase providing the cell with sulfate from arylsulfate esters found in decaying vegetative matter.

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Sulfur Metabolism of *Aerobactor aerogenes* II. The Purification and Some Properties of a Sulfatase

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An enzyme which catalyzes the hydrolysis of *p*-nitrophenylsulfate has been extensively purified from derepressed cultures of *Aerobacter aerogenes*, strain 9621 (American Type Culture Collection). The sulfatase activity of the organism is entirely an expression of this enzyme. The preparation is shown to be homogeneous in the ultracentrifuge and to have a molecular weight of approximately 41,000. A number of physical properties useful for the characterization of the enzyme have been determined. Novel properties of the sulfatase include an anomalous hydrolysis rate-temperature profile and a substrate-dependent inactivation by cyanide.

An enzyme having phenolsulfatase activity may be defined as one which catalyzes the hydrolytic cleavage of sulfate monoesters of phenols to the parent phenol and inorganic sulfate. Despite the fact that the biochemical literature contains many reports of studies on crude and purified preparations of phenolsulfatases from a variety of sources (for summarizing references see Gregory and Robbins, 1960; Dodgson and Spencer, 1956b), no clear understanding of the physiological role played by this family of enzymes has emerged. A major difficulty in this regard has been the unavailability of a homogeneous enzyme protein obtainable from a well-characterized organism such as a bacterium, in which the pathways of sulfur metabolism are amenable to investigation. Dodgson *et al.* (1955) have

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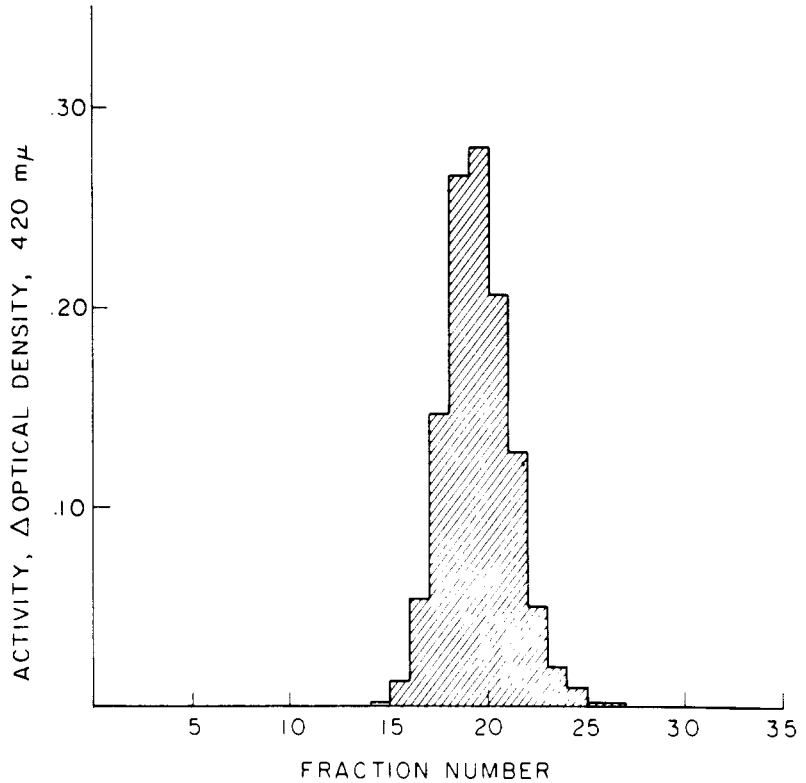

FIG. 1.—Distribution of *p*-nitrophenylsulfatase activity in a sucrose density gradient. The purified enzyme (4.4 μg) in 0.2 ml 0.01 M Tris-chloride, *p*H 7.5, was layered on the top of a sucrose gradient (5–25% sucrose w/v) formed in 0.01 M Tris-chloride, *p*H 7.5, in the manner described by Pazur *et al.* (1962), in a Lusteroid centrifuge tube (13 × 50 mm). The tube was centrifuged 17 hours at 39,000 rpm in a Spinco SW-39 rotor. At the end of this time thirty-six fractions were collected by puncturing the bottom of the tube and allowing drops to form at a uniform rate by controlling air inflow at the top of the tube. Aliquots of all fractions were assayed for *p*-nitrophenylsulfatase activity in the standard way. Activity is expressed as optical density change at 420 mμ/20 μl aliquot per 10 minutes.

purified a phenolsulfatase from the bacterium *Alcaligenes metalcaligenes* but have not demonstrated the degree of homogeneity of their preparation.

Harada and his collaborators (Harada and Kono, 1954; Harada, 1957) have studied the nutritional requirements for the production of phenolsulfatase by *Aerobacter aerogenes*, however no attempts to purify the enzyme have been published.

In connection with our studies on the mechanism of control of enzyme synthesis described in the accompanying communication (Rammler *et al.*, 1964) we have extensively purified and partially characterized the phenolsulfatase of *A. aerogenes*, strain 9621. The enzyme appears to be specific for the sulfate esters of simple phenols. It is synthesized by the bacterium, when grown in minimal salts medium with glycerol as sole carbon source, only under conditions of sulfate deprivation (Rammler *et al.*, 1964). This paper describes a number of properties of the purified enzyme and the procedure for its isolation. To our knowledge it constitutes the first report of the isolation of a sulfatase enzyme in virtually homogeneous form.

MATERIALS AND METHODS

General.—Potassium *p*-nitrophenylsulfate was synthesized essentially according to Burkhardt and Lapworth (1926) and was purified by recrystallization from water. Potassium phenolphthalein-disulfate and potassium α-naphtholsulfate were prepared by the methods described by Whitehead *et al.* (1952) and were also purified by recrystallization from water. Streptomycin sulfate was California Corp. for Biochemical Research "B" grade. Potassium *p*-nitrophenylphosphate was obtained from the Sigma Chemical Co., and other sulfatase substrate analogs were products of Eastman Kodak.

Cellulose ion exchangers were purchased from Serva Entwicklungslabor and were thoroughly washed with 2 N KOH before use.

Unless otherwise noted, all procedures were carried out at 0–3°. Centrifugation steps involved in the purification of the enzyme were normally carried out at 9000 rpm in a Servall refrigerated centrifuge, Model RC-2.

Enzyme Assay.—Cell-free preparations and purified enzyme samples were routinely assayed by the following modification of published procedures (Dodgson and Spencer, 1957): appropriate quantities of the enzyme contained in 10–20 μl were added to a cuvet containing, in a final volume of 1 ml, 250 μmoles Tris-acetate,[1] *p*H 7.1 and 8 μmoles NPS. The rate of formation of *p*-nitrophenol was calculated from the observed increase in absorbancy at 420 mμ, while the reaction mixture was incubated at 38°. A molar extinction coefficient of 6750 at 420 mμ, for *p*-nitrophenol at a *p*H of 7.1, was used for the calculation of specific activity. Specific activities are expressed as μmoles of *p*-nitrophenol formed from NPS per minute per mg of protein contained in the reaction cuvet. Changes in absorbancy at 420 mμ were continuously recorded using a Cary spectrophotometer, Model 15. Temperature during the assay was controlled using a Haake thermostated circulating pump.

[1] Abbreviations used in this work: NPS, *p*-nitrophenylsulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; DEAE-, diethylaminoethyl-; TEAE-, triethylaminoethyl-.

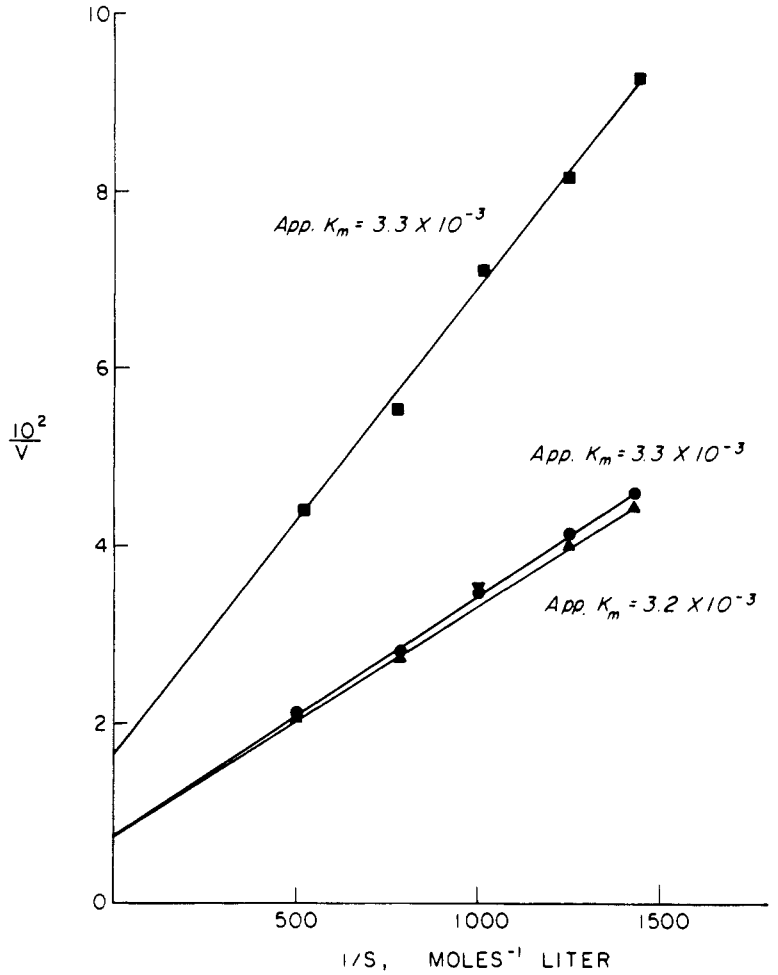

FIG. 2.—Enzymic rate of hydrolysis of NPS as a function of substrate concentration, plotted according to Lineweaver and Burk (1934). Assays were carried out in the usual manner at 38°, as described in the text. The two lower curves give the data of duplicate experiments using a fresh preparation of the enzyme, while the upper curve was obtained using a preparation which was several weeks old. The specific activity, v, is expressed as μmoles p-nitrophenol formed per minute per mg of protein in the cuvet.

Protein was estimated by the biuret method (Gornall *et al.*, 1949) or the Lowry method (Lowry *et al.*, 1951) with bovine plasma albumin as standard.

Purification of the Enzyme.—Aerobacter aerogenes strain 9621 (American Type Culture Collection) was grown at 28° for 20 hours with moderate aeration, in a medium of the following composition: NaCl, 0.1 M; NH_4Cl, 0.01 M; $MgCl_2$, 0.001 M; $CaCl_2$, 10^{-6} M; KPO_4 buffer, *p*H 7.0, 0.1 M, and glycerol, 10 g/liter. Resting-phase cells were harvested from 300 liters of culture medium, yielding 2.2 kg of bacterial paste.

The above paste (500 g) was suspended in sufficient 0.01 M Tris-chloride, *p*H 7.5, to give a final volume of 1 liter. The cells were ruptured either by extrusion from a French pressure cell under a pressure of 8–9 tons or by treatment in 100-ml batches with a 20-kc sonifier (Branson Instruments Inc., Stamford, Conn.) at full power setting for 8 minutes. The cell debris was centrifuged 10 minutes at 30,000 rpm in the No. 30 rotor of a Spinco Model L ultracentrifuge, then washed once with 500 ml of 0.01 M Tris-chloride, *p*H 7.5. The protein concentration of the combined supernatants was adjusted to 25 mg/ml by addition of 0.01 M Tris-chloride, *p*H 7.5.

To precipitate nucleic acids, a 10% (w/v) solution of streptomycin sulfate was added to the above extract (0.1 ml/ml extract). After 10 minutes the precipitate was removed by centrifugation. The clear supernatant was quickly warmed to 35° in a 50° water bath, and 95% ethanol at −10° (0.67 ml/ml) was rapidly added with efficient stirring. A copious precipitate of inactive protein was immediately formed. The temperature was again raised to 35° and kept there for 2 minutes. Following this the mixture was chilled as rapidly as possible (in an ice-salt bath) to 2° and more cold 95% ethanol (0.58 ml/ml streptomycin supernatant) was added, lowering the temperature to −2°. The mixture was maintained at this temperature for 5 minutes. The precipitate, now containing nearly all the sulfatase activity, was then collected by centrifugation. The pellet was extracted by stirring for 15 minutes with Tris buffer, *p*H 7.5, and the clear extract was fractionated with ammonium sulfate as follows: Solid ammonium sulfate (0.5 g/ml extract) was added and the mixture was stirred for 20 minutes. The protein precipitate, along with undissolved ammonium sulfate, was removed by centrifugation. Saturated ammonium sulfate solution (20°, 0.4 ml/ml) was added to the supernatant and after 10 minutes the precipitate was collected, dissolved in a small volume of 0.01 M Tris-chloride, *p*H 7.5, and dialyzed against several changes of the same buffer. After the removal of a small amount of insoluble material, the dialyzed fraction was chromatographed on a column (18 × 200 mm) of DEAE-cellulose. Elution was carried out with a linear gradient of 0–0.5 M NaCl in 0.01 M Tris-chloride, *p*H 7.5 (800 ml), at a rate of 1.5 ml/minute. Fractions containing sulfatase

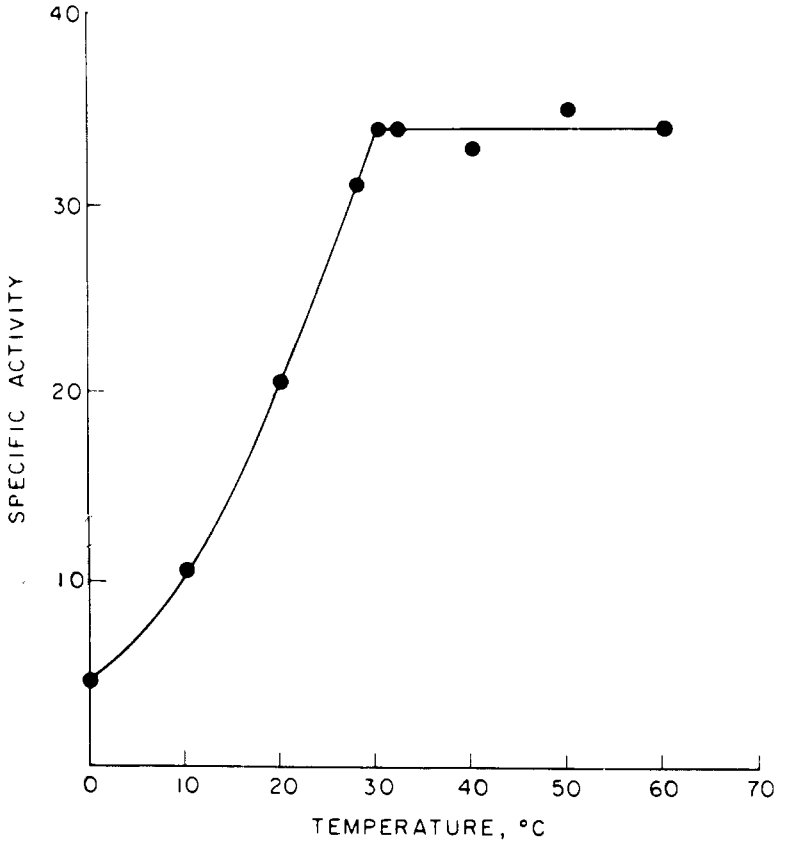

FIG. 3.—Temperature dependence of the enzyme-catalyzed rate of hydrolysis of NPS. Assays were carried out as described in the text, by addition of 20-μl aliquots of a solution containing the enzyme to 1 ml of the assay mixture, previously equilibrated at the desired temperature. Specific activities are expressed as μmoles *p*-nitrophenol formed per minute per mg of protein in the cuvet.

activity appeared as a single peak after 150 ml of the effluent had been collected. The three fractions having the highest specific activity were combined and further purified by elution from a column of TEAE-cellulose (12 × 250 mm) with a linear gradient of 0–0.5 M NaCl in 800 ml of 0.02 M Tris-chloride, *p*H 8.2, using a flow rate of 0.5 ml/minute. A single, isolated peak of sulfatase activity was eluted at an effluent volume of *ca.* 220 ml. Several fractions which showed identical specific activity were combined and concentrated as desired by lyophilization.[2]

Attempts to achieve further purification of the sulfatase have not yielded fractions with higher specific activity. The TEAE-cellulose-chromatographed fraction represents a 350-fold purification of the enzyme and can routinely be obtained in a yield of 10% or greater. The specific activity and the yield of enzyme recovered at each stage of the purification procedure are indicated in Table I.

RESULTS

Molecular Weight and Homogeneity.—The molecular weight of the purified sulfatase was determined by the approach to sedimentation equilibrium method (Archibald, 1947), using a Spinco Model E ultracentrifuge equipped with interference optics. A solution of the enzyme at a concentration of 4.5 mg/ml was thoroughly dialyzed against 0.1 M Tris-chloride, *p*H 7.5, containing 10^{-4} M EDTA. The concentration gradient at the meniscus of the protein solution was calculated from the slope of the interference fringes leaving the meniscus. A molecular weight of $4.07 \times 10^4 \pm 0.11 \times 10^4$ was calculated by means of the equations elaborated by Schachman (1959). Values for the molecular weight obtained at three well-separated times during the run were constant within the limits of error of the method (Table II), indicating homogeneity of the sample with respect to molecular size. Using the same sample in a sedimentation-velocity experiment, a sedimentation coefficient $s_{20,w}$ of 3.5 S and frictional ratio of 1.24 were obtained. The schlieren pattern contained a single, uniform peak throughout the course of the

TABLE I
SUMMARY OF PURIFICATION PROCEDURE

Fraction	Total Protein (mg)	Total Units	Specific Activity[a]
Cell sap	31,200	6550	0.21
Streptomycin	24,700	5900	0.24
Ethanol ppt	2,430	4000	1.64
$(NH_4)_2SO_4$ fractionation	700	2940	4.2
DEAE-cellulose chromatographed	26	1070	41
TEAE-cellulose chromatographed	9	656	73

[a] Expressed as μmoles *p*-nitrophenol formed per minute per mg of protein in the reaction vessel at 38°.

[2] The purification procedure shown in Table I is typical of five similar experiments where the final specific activity varied between 69 and 83 μmoles of *p*-nitrophenol formed per minute per mg of protein in the reaction vessel at 38°. The sulfatase obtained by this method was used in all the characterization experiments. The specific activity of the lyophilized enzyme was variable and less than that of the enzyme obtained directly from the last chromatography step.

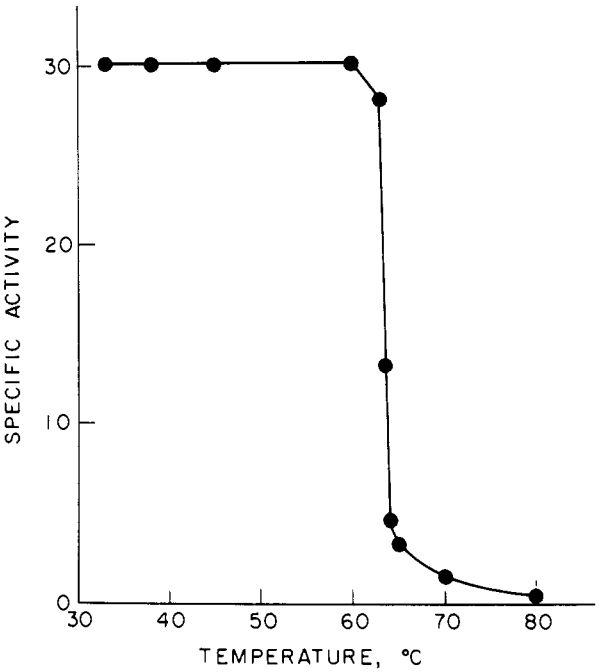

FIG. 4.—Curve illustrating the rapid inactivation of *A. aerogenes* phenolsulfatase at temperatures higher than 60°. Aliquots (20 μl) of a solution containing the enzyme at a concentration of 0.22 mg/ml were added to 1 ml of the assay mixture, previously equilibrated at the temperatures indicated by the points on the curve. Values of specific activity at a time 1 minute after addition of the enzyme were determined for each temperature. Specific activities are expressed as μmoles *p*-nitrophenol formed per minute per mg of protein in the cuvet.

TABLE II
DETERMINATION OF MOLECULAR WEIGHT BY SEDIMENTATION EQUILIBRIUM

Time from Beginning of Run (min)	Mol. Wt.
20	4.10×10^4
40	4.20×10^4
60	3.91×10^4

sedimentation showing the sample to be homogeneous with respect to molecular weight and shape.[3]

Additional evidence that a single molecular species is responsible for sulfatase activity in our preparation was obtained by means of density-gradient centrifugation. Figure 1 describes the results obtained when the purified enzyme was sedimented in a sucrose density gradient according to the method of Martin and Ames (1961) as modified by Pazur *et al.*, (1962). It can be seen that NPSase activity is distributed in a single narrow and symmetrical band near the midpoint of the gradient. Details of the experiment are given in the legend to Figure 1.

The purified enzyme is considered to be free from nucleic acid because the ultraviolet spectrum shows an optical density ratio, 280/260 mμ, of 1.95.

Enzymic Activity and Substrate Concentration.—The assay conditions described in the section on

[3] In one experiment, in which the enzyme was dialyzed against buffer containing 2-mercaptoethanol, a second peak ($s_{20,w}$ = 7.6 S) was observed. As the specific activity of this preparation was the same as that of homogeneous preparations, it appears likely that the 7.6 S protein is an aggregate of the sulfatase.

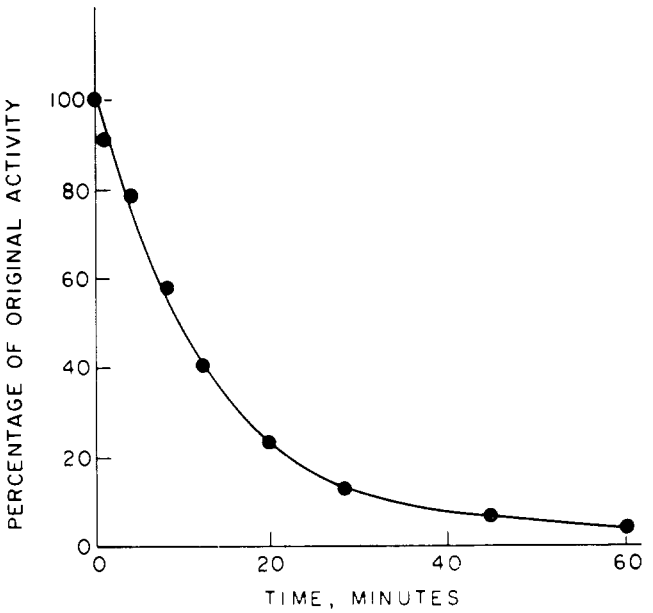

FIG. 5.—Denaturation of *A. aerogenes* phenolsulfatase as a function of time at 60°. The purified enzyme was diluted to a concentration of 0.14 mg of protein per ml in a solution of 0.01 M Tris-chloride, *p*H 7.5, which had previously been equilibrated at 60°. At the times indicated in the figure, 20-μl aliquots were assayed at 37° as described in the text.

methods were chosen so that small variations in ionic strength, substrate concentration, and temperature of the assay medium would not alter the enzyme-catalysed rate of hydrolysis of NPS. The rate is independent of substrate concentration in the range of 6–20 μmoles/ml and independent of buffer concentration at least from 100–300 μmoles/ml. A high concentration is employed because Tris is an inefficient buffer at *p*H 7.1. We have found a *p*H-rate profile for the reaction catalyzed by the purified enzyme with a maximum at *p*H 7.1, virtually the same as that found (Rammler *et al.*, 1964) for the assay of the *p*-nitrophenylsulfatase activity of intact cells.

The kinetics of the reaction are strictly zero order for at least 1 minute at 38° under standard assay conditions, and for longer periods when lower concentrations of substrate are used.[4] The zero-order rate of hydrolysis of NPS is linearly dependent on the amount of enzyme added to the assay cuvet. At low concentrations of NPS the rate shows a linear dependence on substrate concentration. The apparent K_m of the enzyme was determined using both a freshly prepared sample and one which had lost more than half its original activity through aging. As shown in Figure 2, in which the hydrolysis rates measured at several different substrate concentrations are plotted according to the double reciprocal method of Lineweaver and Burk (1934), both preparations gave the same apparent K_m, 3.3×10^{-3}. The value of V_{max} obtained for the fresh preparation is 125 μmoles NPS hydrolyzed/minute per mg protein.

Temperature Dependence of Enzyme Activity.—Figure 3 shows the relationship between the specific activity of the sulfatase and the temperature of reaction. Between 0° and 28° a normal dependence of reaction rate on temperature is observed. A plot of the logarithm of the rate constants against the reciprocal of the absolute temperature over this range gives a straight line with a slope = −2460 °K. Using the Arrhe-

[4] The enzyme is measurably inactivated by incubation at 38° with 0.1 M NPS (see Table V).

nius equation (Arrhenius, 1889) an energy of activation, ΔH_a, for the enzymic hydrolysis of NPS of 11.3 kcal/mole may be calculated. In the range 30–60° the reaction rate is anomalously independent of temperature.[5] At temperatures above 60° the enzyme is rapidly denatured under the conditions of the assay.

Thermolability.—The enzyme loses activity only slowly at temperatures appreciably below 60°. When the specific activity was measured over the temperature range of 30–80° at a time 1 minute after addition of the enzyme to a cuvet at that temperature, the curve shown in Figure 4 was obtained. The curve emphasizes the sharp denaturation point of the enzyme between 60° and 65° and, because it is highly reproducible with different preparations, may be considered a useful criterion for identification of the protein. In particular, this method should be of value in differentiating "wild type" and genetically altered enzymes.

Figure 5 describes the kinetics of thermal denaturation of the sulfatase at 60°. The curve is not described by a rate expression of integral order. Thus far, renaturation of the thermally inactivated enzyme has not been accomplished.

Dependence of Stability on pH.—Figure 6 shows the *p*H-stability profile for the enzyme in dilute solution at 3°. The conditions of the experiment are given in the legend. The inactivations observed at high and low *p*H were not reversible under the conditions used.

Effect of Urea.—The sulfatase is rapidly inactivated in concentrated urea solutions. The rates of inactivation at various temperatures between 0° and 37° are illustrated in Figure 7. The logarithms of the first-order rate constants between 26° and 37° when plotted against the reciprocals of the absolute temperatures give a straight line, from the slope of which an activation energy of denaturation equal to 38 kcal/mole was calculated. The enzyme which has been inactivated in urea regains part of its sulfatase activity on removal of the urea by slow dialysis against 0.01 M Tris-chloride, *p*H 7.5. Recovery of more than 25% of the original *p*-nitrophenylsulfatase activity has not been achieved by this means.

Substrate Analogs.—Like most sulfatases previously reported in the literature, the *A. aerogenes* enzyme appears to have a rather limited substrate specificity. Phenolphthaleindisulfate and α-naphtholsulfate are not hydrolyzed by the enzyme. On the other hand, the rate of hydrolysis of phenylsulfate under the standard assay conditions was determined (Abbot, 1947), and was found to be 52% of the rate of hydrolysis of NPS by the same enzyme sample.

A number of substrate analogs were tested for competitive inhibition of *p*-nitrophenylsulfatase activity. The data of Table III show that the potassium salts of *p*-nitrobenzoic acid, *p*-nitrobenzenesulfonic acid, *p*-nitrobenzenearsonic acid, and *p*-nitrophenylphosphoric acid were not effective competitors when present in concentrations equal to that of the substrate. It should be noted that *p*-nitrophenylphosphate is also completely inactive as a substrate of the enzyme. When *p*-nitrophenylphosphate replaced NPS under standard assay conditions, no change in optical density at 420 mμ was observed during 1 hour of incubation with 20 μg of the purified enzyme. At the end of this time rapid release of *p*-nitrophenol on addition of NPS indicated that no appreciable loss of sulfatase activity had occurred.

Effects of Metals.—The enzyme was assayed in the presence of a number of metal ions. As shown in Table IV, none of the metals at a concentration of 10^{-4} M caused activation of the enzyme above the level of the control. Only zinc and lead effected more than 10% inhibition of *p*-nitrophenylsulfatase activity.

The sulfatase contains no essential metal which is extractable with EDTA. The purified enzyme was dialyzed with internal and external stirring against 100 volumes of 0.01 M Tris-chloride, *p*H 7.5, containing 10^{-4} M EDTA. The dialysate was replaced after 2 hours and dialysis continued for 17 hours. At the end of this time the EDTA-treated enzyme had a specific activity of 27.5 compared with a value of 26 obtained with an identical untreated sample.

Inhibitors.—For comparison of the *A. aerogenes* enzyme with others reported to hydrolyze NPS, the inhibitory effects of phosphate, sulfate, fluoride, and cyanide were investigated. At a concentration of 10^{-2} M, sodium sulfate showed no inhibition of *p*-nitrophenylsulfatase activity, while phosphate at the same concentration gave 55% inhibition, and at 10^{-3} M gave 17% inhibition. Fluoride ion at a concentration of 10^{-2} M gives 67% inhibition of the sulfatase activity but gives only 4% inhibition at 10^{-4} M.

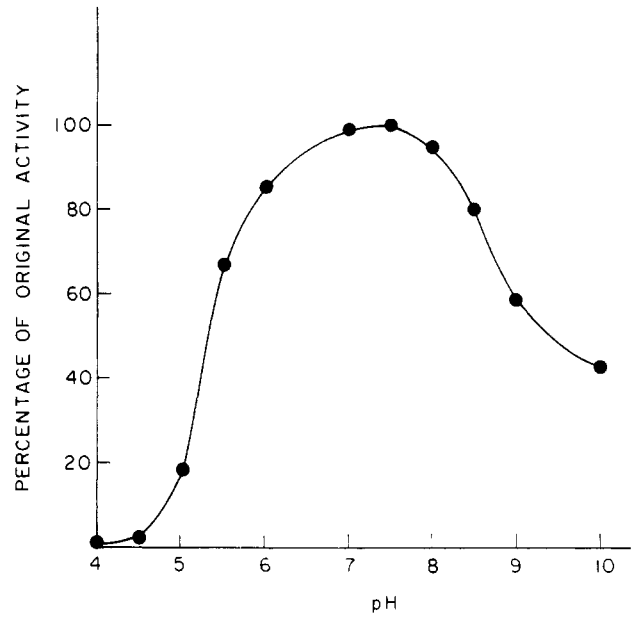

FIG. 6.—Stability of *A. aerogenes* phenolsulfatase as a function of *p*H. Aliquots of purified enzyme, 0.022 mg/ml, were diluted 10-fold in 0.5 M buffers at *p*H values indicated by the points on the curve. Buffers in the *p*H range 4.0–6.0 were sodium acetate, those in the range 7.0–8.5 were Tris-chloride, and those from 8.5–10.0 were sodium glycinate. After 18 hours at 3°, 0.1-ml aliquots were assayed at *p*H 7.1, 38°, in the usual manner, as described in the text.

TABLE III
EFFECT OF SUBSTRATE ANALOGS ON *p*-NITROPHENYLSULFATASE ACTIVITY[a]

Analog	ΔOD_{420}/Minute
—	0.33
p-Nitrobenzoate	0.31
p-Nitrobenzenesulfonate	0.31
p-Nitrobenzenearsonate	0.31
p-Nitrophenylphosphate	0.32

[a] Assays for sulfatase activity were carried out under standard conditions except that both substrate and substrate analogs were present in the assay medium at a concentration of 10^{-3} M.

[5] This observation has been confirmed by several repetitions of the experiment.

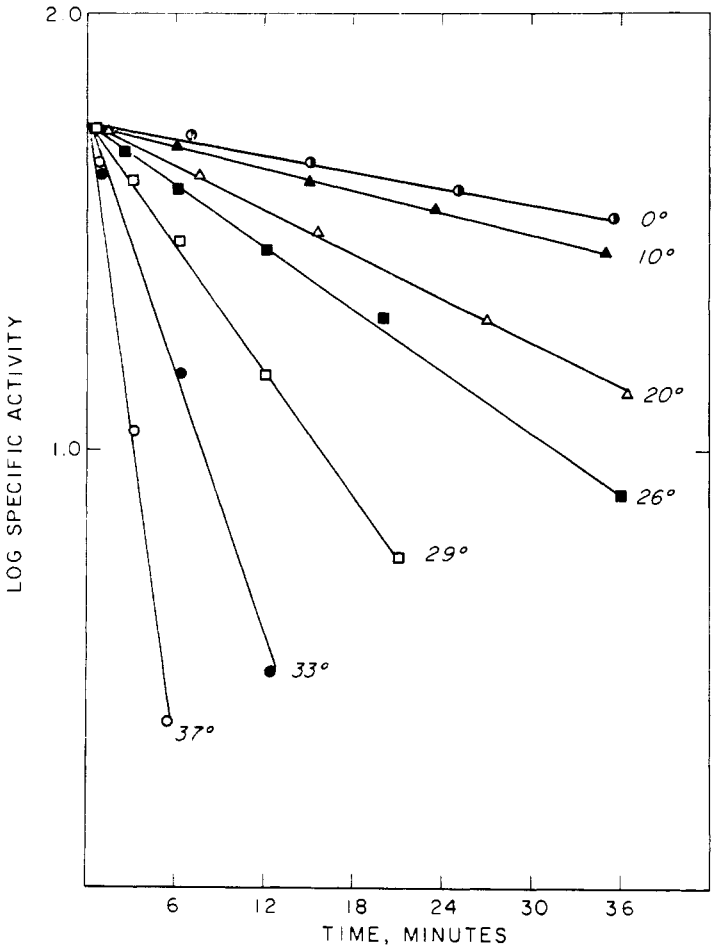

FIG. 7.—First-order rates of inactivation of the sulfatase in 6.5 M urea. Aliquots (0.1 ml) of a solution of the purified enzyme were diluted with 0.4 ml of a solution containing 0.01 M Tris-chloride, *p*H 7.5, and 8 M urea to give a final protein concentration of 0.028 mg/ml. At the times indicated by points on the curves, aliquots (20 μl) of the diluted solutions were assayed for *p*-nitrophenylsulfatase activity, at 38°, in the usual manner. Specific activity is expressed as μmoles of *p*-nitrophenol formed per minute per mg of protein in the assay cuvet.

TABLE IV

INFLUENCE OF METAL IONS ON *p*-NITROPHENYLSULFATASE ACTIVITY[a]

Metal Ion	Specific Activity	Metal Ion	Specific Activity[b]
—	25	Ni^{2+}	24
Mg^{2+}	25	Sn^{4+}	24
Ca^{2+}	25	Ca^{2+}	24
Fe^{2+}	25	Cd^{2+}	23
Co^{2+}	24	Ag^{+}	22
Ce^{2+}	24	Zn^{2+}	20
Ba^{2+}	24	Pb^{2+}	15

[a] Assays were carried out under standard conditions with metals added to give a concentration of 10^{-4} M. [b] Expressed as μmoles of *p*-nitrophenol formed per minute per mg of protein in the reaction vessel at 38°.

TABLE V

INACTIVATION OF SULFATASE BY CYANIDE IN THE PRESENCE OF NPS[a]

Incubation Temperature (°C)	Molar CN	Concentration NPS	Specific Activity[b] 1 min	5 min
38	—	—	30	29
38	0.01	—	27	29
38	—	0.01	26	25
38	0.001	0.01	19	12
38	0.01	0.001	20	19
38	0.01	0.01	10	<1
0	0.01	0.01	24	8

[a] The enzyme was assayed for *p*-nitrophenylsulfatase activity by the standard method after incubation with the indicated reagents for 1 minute and 5 minutes. [b] Expressed as μmoles of *p*-nitrophenol formed per minute per mg of protein in the reaction vessel at 38°.

While inhibition by fluoride is immediate under the assay conditions used, cyanide at a concentration of 10^{-2} M was observed to inactivate the enzyme at a measurable rate. Cyanide is an effective inhibitor only in presence of a high concentration of substrate. While inhibition by fluoride or phosphate is freely reversible by dilution of the inhibitor, inactivation of the enzyme by cyanide in the presence of NPS is not reversible.

The effect of cyanide was studied as follows: Samples of the purified enzyme were incubated at 38° or 0° in 0.1 M Tris-chloride, *p*H 7.5, at a concentration of 0.053 mg/ml. Potassium cyanide and NPS were added to the samples as indicated in Table V. Aliquots of each sample were assayed 1 minute and 5 minutes after additions of cyanide and NPS. The data of the table show that rapid and complete inactivation of the sulfatase is achieved only when both cyanide and NPS are present in relatively high concentration. The last line of the table shows that the rate of inactivation is strongly temperature dependent. When a mixture of

cyanide and NPS which had been incubated at 38° for 10 minutes was added to the enzyme solution, in 0.1 M Tris at 0°, to give a final concentration of 0.01 M of each reagent, inactivation occurred at the same rate as when the reagents were not premixed. The almost totally inactivated sample of line 6 of Table V did not significantly recover activity when diluted 50-fold in the assay mixture minus NPS and incubated at 38° for 10 minutes, and when the assay was initiated by addition of the usual amount of NPS.

Discussion

In order for the studies described in the accompanying paper (Rammler *et al.*, 1964) to be amenable to interpretation it was necessary to show that the activity of only one enzyme (the derivative of a single structural gene) was being measured. Several lines of evidence point to the conclusion that the *p*-nitrophenylsulfatase activity of *A. aerogenes* strain 9621 is present in a single protein moiety. Chromatographic procedures employed in the purification of the sulfatase have never revealed more than one peak with *p*-nitrophenylsulfatase activity. Centrifugation of the purified enzyme in a sucrose density gradient gives only a single symmetrical band of *p*-nitrophenylsulfatase activity. In addition, the physical homogeneity of the preparation has been clearly demonstrated in the analytical ultracentrifuge. Preliminary results with disc electrophoresis in polyacrylamide gels reveal only a single narrow band containing *p*-nitrophenylsulfatase activity. These latter results are not as yet unequivocal, as experiments to date have been carried out at high *p*H values where the enzyme is not maximally stable.[6] In this connection it should be mentioned that, despite the relatively high thermal stability of the enzyme, purified preparations slowly lose activity at variable rates on storage in the cold. The loss in activity parallels the appearance of several additional protein containing peaks in TEAE-cellulose chromatograms of material which had originally composed a single uniform peak, when eluted from the same column. None of these additional peaks shows *p*-nitrophenylsulfatase activity. The Michaelis constant, K_m, of such partially inactivated preparations is identical with that of fresh preparations, indicating that only alteration from native enzyme to totally inactive protein has occurred. The reasons for this instability are now being investigated.

Enzymes showing hydrolytic activity toward NPS have been classified by Dodgson and Spencer (1957) as type 1 and type 2 on the basis of their relative affinities for NPS and nitrocatecholsulfate, as well as their inhibition by fluoride, cyanide, phosphate, and sulfate. The enzymes of type 1 are inhibited by cyanide only, while those of type 2 are inhibited by fluoride, phosphate, and sulfate, but not by cyanide. As the enzyme described here is inhibited by fluoride, cyanide, and phosphate, but not by sulfate, it does not fall into either category.

Dodgson and Spencer (1956a) have shown that the inhibition by cyanide of *p*-nitrophenylsulfatase activity of the sulfatase of *A. metalcaligenes* is of the rare "anticompetitive" type, being dependent on the presence of NPS. The irreversible cyanide inhibition of the *A. aerogenes* sulfatase also occurs only in the presence of substrate. The relatively slow rate of the inactivation suggests that reaction of a covalent bond is involved, such as the cleavage of a disulfide linkage (Fraenkel-Conrat, 1941). Studies designed to elucidate the mechanism of the cyanide inhibition are currently being pursued in this laboratory.

The rather remarkable behavior of the enzyme-catalyzed hydrolysis rate of NPS as a function of temperature merits some comment. Although the activation energy (11.3 kcal/mole) of the reaction in the temperature range of 0–28° is normal for a hydrolytic enzyme, the apparent lack of temperature dependence in the range of 30–60° and the sharp break in the rate-temperature profile in the vicinity of 28° are puzzling. Such transitions have been interpreted by Sizer (1943) as representing a change in conformation of the catalytic protein. On the other hand, it has been claimed (Kistiakowsky and Lumry, 1949) that sharp breaks in Arrhenius plots for enzyme-catalyzed reactions are artifacts, and should not be given significant interpretation. The present case represents a much more striking deviation from ideal enzyme kinetics than those discussed by the above authors, and is not amenable to trivial explanation. An understanding of the phenomenon must await the results of further experiments.

[6] Electrophoresis at *p*H 9.2 in a gel of *ca.* 35 A mesh size shows, in addition to the sharp band with *p*-nitrophenylsulfatase activity, an inactive protein band which remains at the origin of the lower gel, and which we ascribe to denatured or polymerized enzyme protein.

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