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Arylsulfatase in *Salmonella typhimurium*: Detection and Influence of Carbon Source and Tyramine on Its Synthesis

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Arylsulfatase synthesis was shown to occur in *Salmonella typhimurium* LT2. The enzyme had a molecular weight of approximately 50,000 and was separated into five forms by isoelectrofocusing. The optimal pH for substrate hydrolysis was pH 6.7, with Michaelis constants for nitrocatechol sulfate and nitrophenyl sulfate being 4.1 and 7.9 mM, respectively. Enzyme synthesis was strongly influenced by the presence of tyramine in the growth medium. The uptake of [^{14}C]tyramine and arylsulfatase synthesis were initiated during the second phase of a diauxie growth response, when the organism was cultured with different carbon sources. Adenosine 3',5'-cyclic monophosphoric acid enhanced the uptake of tyramine and the levels of arylsulfatase synthesized. However, the addition of glucose and glycerol to organisms actively transporting tyramine and synthesizing enzyme caused a rapid inhibition of both of these processes. This inhibition was not reversed by adding adenosine 3',5'-cyclic monophosphoric acid. The results suggest that the effect of the carbon source on tyramine transport and arylsulfatase synthesis may be explained in terms of inducer exclusion.

The study of arylsulfatase in a number of gram-negative bacteria has shown these enzymes to be repressed by compounds in the proposed pathway to cysteine biosynthesis (sulfate, sulfite, sulfide) and derepressed by taurine and methionine (3, 7, 8, 11, 12, 15, 17). Extensive investigation with one organism, *Aerobacter aerogenes*, has demonstrated additional control via catabolite repression with glucose (14), nitrogen repression with NH_4Cl (14), and relief from sulfate-mediated repression by tyramine (2). Further studies on the regulation of arylsulfatase synthesis were undertaken with the taxanomically closely related *Klebsiella aerogenes*. This organism was chosen in view of the fact that *A. aerogenes* lacked a suitable genetic exchange system (22) and that little or no arylsulfatase was produced by *Escherichia coli* and *Salmonella typhimurium*, the bacteria most widely used in genetic analysis (1). From this work a model was proposed implicating sulfate, tyramine oxidase, and ammonium as possible elements regulating arylsulfatase synthesis in *K. aerogenes* (18, 20, 21).

Recently, these workers reported on the formation of intergeneric hybrids arising from transfer of arylsulfatase genes from *K. aerogenes* to *E. coli* and subsequently to *S. typhimurium*. It was suggested that the system for regulation has been conserved more than the structure or function of the enzyme in enteric bacteria (19).

In this investigation we show that, despite repeated claims of these workers to the contrary (1, 19, 32), *S. typhimurium* LT2 does synthesize significant levels of arylsulfatase. This synthesis was directly related to the presence of tyramine, was not subject to nitrogen repression, and was inhibited by a variety of carbon sources. This latter effect did not appear to result from metabolism of glucose and may be explained as a tyramine exclusion phenomenon directly related to transport mechanisms.

This raises the question as to whether the collective control elements proposed by Murooka et al. (18) are common to gram-negative bacteria in general or are unique to *K. aerogenes*. More importantly, the interpretation of the latest genetic work with *K. aerogenes-S. typhimurium* (19) now may be the subject of reappraisal.

MATERIALS AND METHODS

Organisms and cultural conditions. The strains of *S. typhimurium* used in this work were obtained from the following sources: *S. typhimurium* LT2 from K. E. Sanderson, *S. typhimurium* TA3336 *cya* (adenylate cyclase) from M. H. Saier, Jr., and *S. typhimurium* TA3303 *crp* (cAMP receptor protein) from B. N. Ames.

The basal medium contained the following in 1 liter of distilled water: K_2HPO_4, 7 g; KH_2PO_4, 3 g; $MgCl_2$, 0.1 g; K_2SO_4, 0.2 g; $Na_3C_6H_5O_7 \cdot 2H_2O$, 0.5 g; NH_4Cl, 1 g; and glucose, 2 g. Tyramine and all sugars used were

sterilized by passage through 0.45-μm-pore-size membrane filters (Millipore Corp.) and were added to basal medium by aseptic technique. Any modifications of the medium are stated in the text.

The bacteria were grown in 250-ml flasks, containing 50 ml of medium, at 37°C and 150 rpm in a New Brunswick controlled environmental shaker. Growth was monitored by measuring the turbidity of the culture at 620 nm with a Beckman DB spectrophotometer.

Chemicals. The following chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.: nitrocatechol sulfate, *p*-nitrophenyl sulfate, tyramine, *N*-acetyl neuramin lactose, *E. coli* alkaline phosphatase, human albumin, bovine serum albumin, equine myoglobin, catalase, RNase, ovalbumin, cytochrome *c*, immunoglobulin, and cAMP. Ampholytes for isoelectrofocusing and the protein assay kit were obtained from Bio-Rad Laboratories, Richmond, Calif. Peptides with specified molecular weights were obtained from BDH, Toronto, Ontario, Canada, and ICN Pharmaceuticals, Inc., Montreal, Quebec, Canada, was the source of *Clostridium perfringens* neuraminidase. Radioactivity labeled [^{14}C]tyramine hydrochloride was supplied by Amersham-Searle, Arlington Heights, Ill., whereas 3-*O*-methyl-D-glucopyranose was from Calbiochem, La Jolla, Calif. All other chemicals were of the highest grade commercially available and were used without further purification.

Buffer. In this work, Tris buffer refers to tris(hydroxymethyl)aminomethane-maleic acid buffer, 0.05 M, pH 6.7. Unless otherwise stated, this buffer was used throughout the course of the investigation.

Protein determination. Protein concentrations were estimated with the Bio-Rad protein assay kit using Coomassie brilliant blue G-250–perchloric acid reagent (25) and bovine serum globulin as the standard.

Enzyme assay. Routinely, arylsulfatase in whole cells was measured by a modification of the method of Dodgson and Spencer (7) in which a 0.2-ml sample of standard bacterial suspension (optical density at 620 nm, 0.45; approximately 5×10^8 cells per ml) in Tris buffer was incubated with 0.2 ml of 20 mM substrate, either 5-nitrocatechol sulfate (NCS) or *p*-nitrophenyl sulfate (NPS) at 37°C for 5 to 60 min. The reaction was terminated by the addition of 0.8 ml of either 1 N or 0.2 N NaOH when NCS and NPS were the respective substrates. Liberated nitro-phenolate ion was measured spectrophotometrically at 515 nm (nitrocatechol) or 410 nm (nitrophenol). Under the conditions of assay, the hydrolysis of substrates was linear with respect to time and enzyme concentration. A unit of enzyme is defined as the amount of enzyme catalyzing the liberation of 1 μg of product per h. Results are reported as specific activities, units of enzyme per milliliter of standard cell suspension, or units per milligram of protein.

Preparation of enzyme extract. The organism was grown and harvested, and an acetone powder was prepared essentially as described by Delisle and Milazzo (6). Enzyme was partially purified by ammonium sulfate precipitation (30 to 90% saturation), filtration with Amicon XM300, XM100, PM50, and PM10 membranes, and chromatography with Sephadex G200.

Isoelectric focusing in gels. Isoelectric focusing, as described by Vesterberg (29), was performed using 7.5% polyacrylamide gels containing 1% ampholytes, pH range 5 to 7, and 0.7% ampholytes, pH range 3 to 10, to stabilize the gradient. After focusing at 200 V for 4 to 6 h, the pH gradient was measured with an Ingold model ES-47310-2 surface microelectrode fitted to a Fisher Acumet model 220 pH meter. Arylsulfatase was visualized by incubation of gels in NCS or NPS, and the pI was estimated by comparison of the band location with the measured pH gradient. Compounds of known pI (RNase, pI 7.8; myoglobin, pI 7.0; catalase, pI 5.6; albumin, pI 4.6; and bromophenol blue, pI 3.2 [26]) run simultaneously in the presence and absence of extract served as controls.

Neuraminidase treatment. The activity of the commercially prepared *C. perfringens* enzyme was tested using the substrate *N*-acetyl neuramin lactose as specified by the manufacturers, and the liberated sialic acid was measured as specified by Warren (30). The method of Stevens et al. (28) was used to determine the effect of neuraminidase treatment on the electrophoretic mobility and electrofocusing pattern of *S. typhimurium* arylsulfatase.

Molecular weight determination. The molecular weight of the active enzyme was determined by the method of Hedrick and Smith (16) by use of the following standards: alkaline phosphatase (80,000), myoglobin (17,000), ovalbumin (43,000), and bovine serum albumin (68,000). The method of Weber et al. (31) was used to estimate the molecular weight in the presence of sodium dodecyl sulfate with bovine serum albumin (68,000), cytochrome *c* (11,700), gamma globulin (50,000), myoglobin (17,000), and ovalbumin (43,000) as standards (26). In addition, this method was performed with commercially prepared peptide standards with molecular weights of 14,300, 28,000, 42,900, 57,200, and 71,500. Arylsulfatase for sodium dodecyl sulfate molecular weight determinations was obtained by electrophoresing enzyme extracts in standard polyacrylamide gel electrophoresis (6) and eluting the active bands from acrylamide slices. Substrate was removed by dialysis, and enzyme was concentrated by Amicon filtration (PM10).

Measurement of the uptake of [^{14}C]tyramine. The uptake of [^{14}C]tyramine (specific activity 50 mCi/mmol) by cultures was measured by removing 0.5-ml samples at appropriate times during growth. These were filtered through membrane filters (0.45-μm pore size; Millipore Corp.), washed twice with 3 ml of basal medium, and counted in a Beckman LS-255 liquid scintillation counter. The values obtained, kilocounts per minute per milliliter, were converted to kilocounts per minute per milligram (dry weight) of cells using a standard curve relating optical density (620 nm) and dry weight.

RESULTS

Characteristics of the arylsulfatase synthesized by *S. typhimurium* LT2. The characteristics of *S. typhimurium* LT2 arylsulfatase

as determined by us were as follows. The molecular weights of intact enzyme and of sodium dodecyl sulfate-treated enzyme were 55,700 ± 4,600 and 53,500 ± 4,700, respectively. The optimal pH values for substrate hydrolysis with NCS and NPS as substrate were 6.7 and 6.7, respectively. The K_m values (estimated from Lineweaver-Burk plots, i.e., 1/v versus 1/[s]) with NCS and NPS as substrate were 4.1 and 7.9 mM, respectively. The isoelectric points (enzyme focused as five isoenzymes) were pH 6.0, 6.2, 6.3, 6.4, and 6.6 (All of these values were obtained using partially purified extract and methodology as described.) These characteristics of the enzyme show that it is directly comparable to other bacterial arylsulfatases in respect to size (6, 22, 24), optimal pH for substrate hydrolysis (10, 22, 24), and kinetic properties (10, 22, 24). It should be noted that, even though the enzyme migrated as a single band in polyacrylamide gel electrophoresis with 7 to 12% acrylamide concentrations, it was separated by isoelectrofocusing into five distinct forms with isoelectric points ranging from pH 6.0 to 6.6. This finding is consistent with reports of the occurrence of arylsulfatase isoenzymes in some bacteria (7, 10), fungi (5), and mammals (28).

Arylsulfatase synthesis in *S. typhimurium*. Since others were unable to detect arylsulfatase synthesis in this bacterium (2, 19, 32), it was important to determine precisely when the enzyme was formed during growth of the organism. It was interesting to find that enzyme synthesis was dependent on tyramine. Figure 1A shows that, in the absence of this amine, only low levels of enzyme were detected and, with the addition of 10 mM tyramine (Fig. 1B, C, and D), growth was biphasic and enzyme synthesis occurred at the onset of the second phase. Routinely, 10 mM tyramine was used since it was found (unpublished data) that high levels of enzyme were detected at this concentration. The initiation of the second growth phase and arylsulfatase synthesis were progressively delayed from 300 to 420 min when the concentration of glucose was increased from 0.1 to 0.4%. Once initiated, the rate of synthesis and maximum levels of enzyme detected were essentially the same. This growth pattern was indicative of a diauxie phenomenon and may have resulted from the delayed use of tyramine as a carbon source. It should be stressed at this point that the same pattern was observed also with glycerol, xylose, and arabinose (data not shown).

Uptake of [^{14}C]tyramine and arylsulfatase synthesis in three strains of *S. typhimurium*. One possible explanation for the delay in enzyme synthesis was the inability of the organism to take up tyramine during the initial phase of growth. This possibility was examined

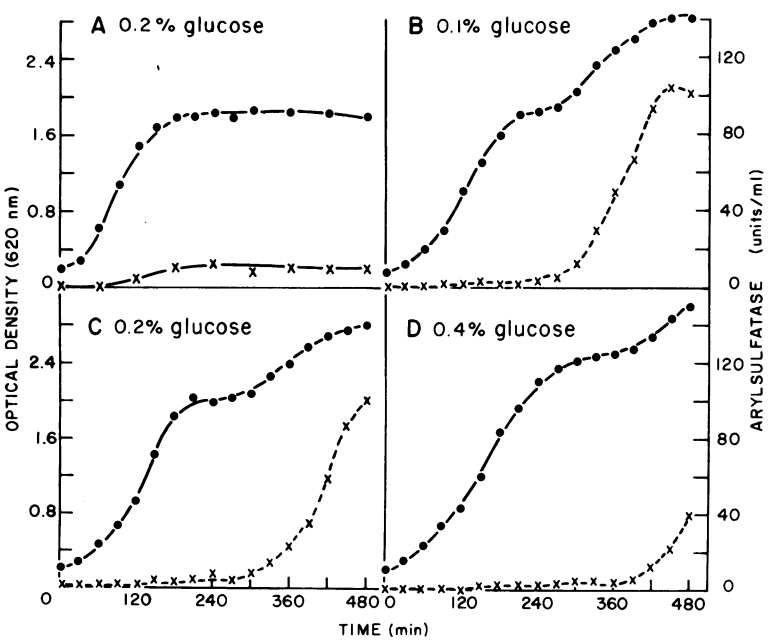

FIG. 1. *Effect of tyramine and concentration of glucose on arylsulfatase synthesis in S. typhimurium. The organism was grown in basal medium without tyramine (A) and with 10 mM tyramine and different concentrations of glucose (B, C, D). Symbols: ●, growth; ×, arylsulfatase.*

by using [^{14}C]tyramine. It was evident (Fig. 2A) that, in the wild-type organism, entrance of tyramine and the increase in enzyme levels were initiated at the beginning of the second period of growth. An obvious assumption would be that glucose may have prevented tyramine transport by cAMP-mediated catabolite repression. When an adenylate cyclase mutant was used (Fig. 2B), the amount of tyramine which entered the cell and the levels of enzyme detected were only one-third of those in the wild type, even though growth was similar. This same pattern, but with reduced levels, was apparent when a cAMP receptor protein mutant was examined (Fig. 2C). Therefore, mutants lacking either adenylate cyclase or cAMP receptor protein synthesized lower levels of arylsulfatase and were impaired in the ability to take tyramine into the cell. These results were consistent with the explanation that catabolite repression influenced both tyramine transport and arylsulfatase synthesis.

Effect of cAMP on tyramine transport and enzyme synthesis. In view of the possible involvement of glucose-mediated catabolite repression, an attempt was made to reverse this effect by the addition of cAMP. Since preliminary findings showed that 5 mM cAMP supported high levels of enzyme synthesis, this concentration was used to study its influence on tyramine transport. When cAMP was added to the wild type (Fig. 3A) and to a *cya* mutant (Fig. 3B), growth was no longer biphasic, tyramine entrance and enzyme synthesis were initiated sooner, and higher levels of both were observed. The response of the *crp* mutant (Fig. 3C) to cAMP was not pronounced. This overall pattern would be expected if catabolite repression were operative. Curiously, however, this explanation failed to account for the previously noted delaying influence of increasing concentrations of glycerol (non-catabolite repression promoting sugar) on enzyme synthesis. In fact, parallel experiments with glycerol and cAMP (Fig. 4A and B) gave the same results as were obtained with glucose and cAMP. Therefore, it seemed that the role of cAMP could not be explained solely in terms of glucose-mediated catabolite repression.

An alternate explanation might involve a direct effect of glucose and glycerol on tyramine uptake and enzyme synthesis. In support of this, it was noted (Fig. 5) that the addition of glucose and glycerol to cells growing with tyramine as a carbon source resulted in a rapid cessation of tyramine uptake and enzyme synthesis and that the addition of cAMP did not reverse this inhibition. It should be stressed that the addition of glucose and glycerol did not alter the growth rate during the experimental period. Furthermore, the non-metabolizable glucose analog, 3-*O*-methyl-D-glucopyranose, was found to produce an identical response, establishing that the metabolism of glucose was not required for the inhibitory action (Fig. 6).

Influence of NH_4Cl and sulfur source on arylsulfatase synthesis. Since NH_4Cl concentration and sulfur source influenced arylsulfatase synthesis in *K. aerogenes*, an attempt was

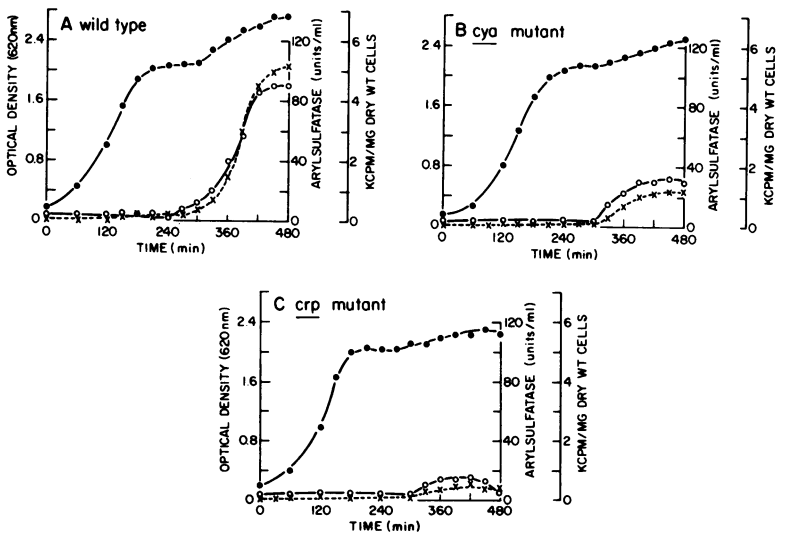

FIG. 2. *Growth, enzyme synthesis, and uptake of [^{14}C]tyramine in three strains of S. typhimurium. The organisms were grown in basal medium containing 10 mM tyramine (including [^{14}C]tyramine). Symbols: ●, growth; ×, arylsulfatase, ○, [^{14}C]tyramine.*

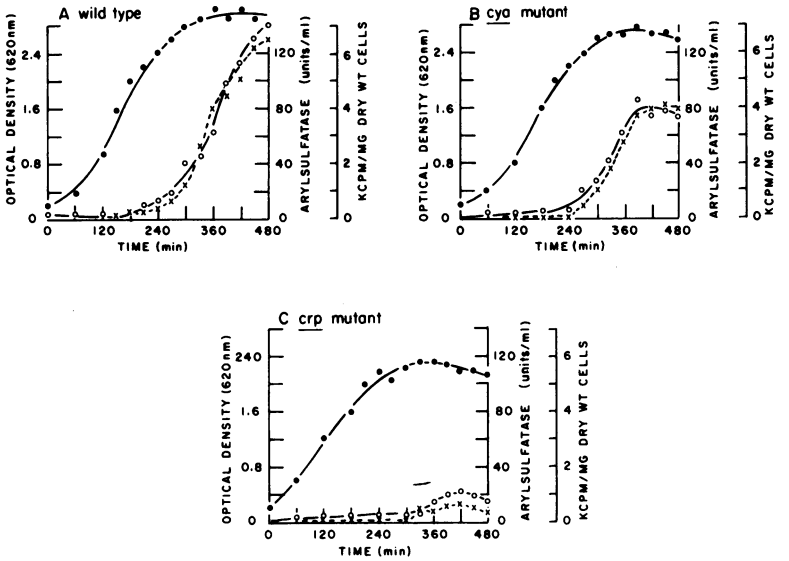

FIG. 3. *Effect of cAMP addition on growth, uptake of [^{14}C]tyramine, and enzyme synthesis in three strains of S. typhimurium. Organisms were grown in basal medium with 10 mM tyramine and 5 mM cAMP. Symbols: ●, Growth; ×, arylsulfatase; ○, [^{14}C]tyramine.*

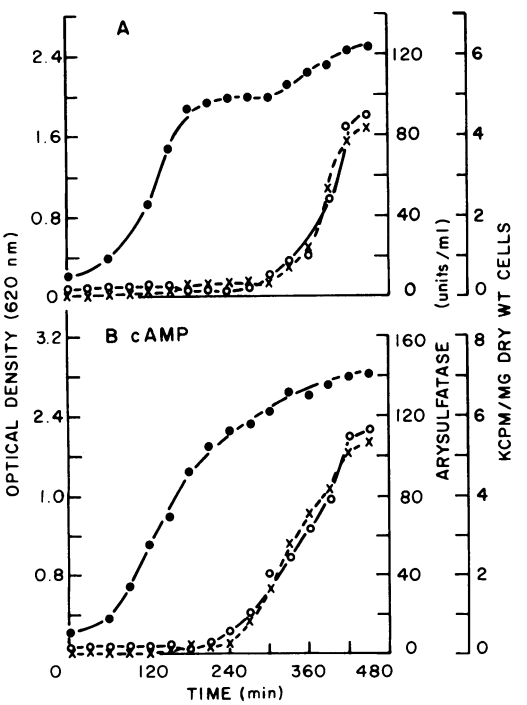

FIG. 4. *Influence of cAMP on growth, [^{14}C]tyramine transport, and enzyme synthesis in S. typhimurium grown with 0.2% glycerol as the carbon source. Symbols: ●, Growth; ×, arylsulfatase; ○, [^{14}C]tyramine.*

made to establish whether these factors affected enzyme synthesis in *S. typhimurium*. Table 1 shows that varying initial concentrations of NH_4Cl from 0.05 to 0.3% had no repressive effect on enzyme synthesis. The low value obtained (0.025% NH_4Cl) reflects the fact that, at concentrations of 0.025% and below, cell growth was negligible. Similarly, the addition of NH_4Cl during the period of enzyme synthesis did not alter the levels of arylsulfatase detected (data not shown). These results show that NH_4Cl did not repress arylsulfatase synthesis in *S. typhimurium*.

It was interesting to find that, when the organism was grown with sulfur sources considered to be repressors (cysteine, sulfate) and a depressor (methionine), enzyme synthesis was directly related to the presence of tyramine. The difference in amounts of enzyme obtained with the three compounds probably results because sulfate was a more readily used source of sulfur.

DISCUSSION

The experiments reported here clearly demonstrate that *S. typhimurium* produces arylsulfatase at levels high enough to permit its purification and use in biochemical and physiological studies. It follows that this organism can now serve in further elucidation of the genetic basis of the control of arylsulfatase synthesis in bacteria.

It is noteworthy that this enzyme, like those

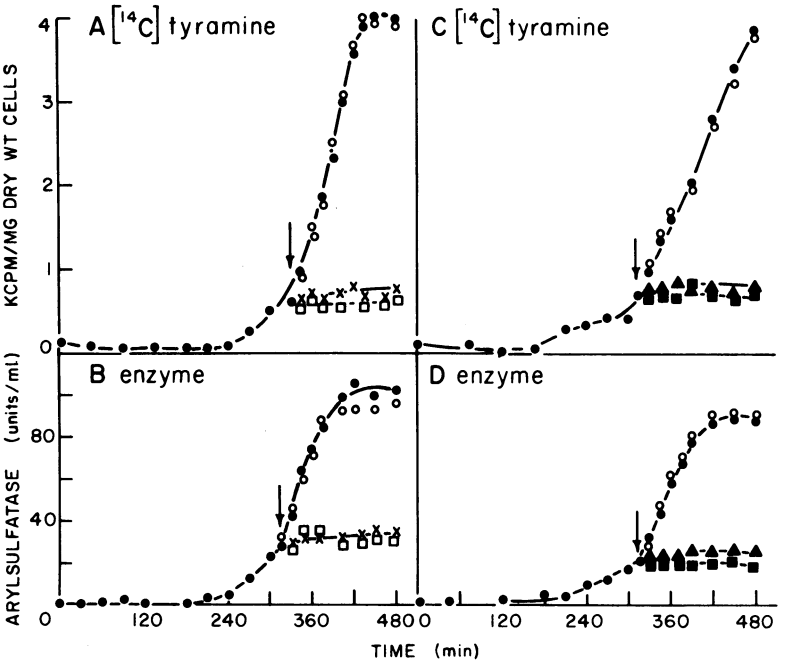

FIG. 5. *Effect of adding glucose and glycerol to S. typhimurium actively synthesizing arylsulfatase when grown in basal medium with tyramine as the carbon source. Additions were made at the time indicated (↓). Symbols: ●, No addition; ○, 5 mM cAMP; ×, 0.2% glucose; □, 0.2% glucose with 5 mM cAMP; ▲, 0.2% glycerol; ■, 0.2% glycerol plus 5 mM cAMP.*

from *Proteus rettgeri* (17), *Pseudomonas aeruginosa* (7), some fungi (5), and mammals (28), was separated into multiple forms. In the case of human arylsulfatase A (28), it was shown by neuraminidase treatment that the isoenzymes differed in charge due to varying amounts of sialic acid in terminal positions. However, similar treatment of *S. typhimurium* arylsulfatase did not alter either the mobility of the enzyme in polyacrylamide gel electrophoresis or its isoelectric focusing pattern. Therefore, the basis for these isozymes does not appear to be the presence of hydrolyzable sialic acid residues, and their origin and function remain unknown.

The major finding of this investigation was the strong influence of tyramine on arylsulfatase synthesis. Studies with other bacteria have shown that, although this compound enhanced enzyme synthesis in *A. aerogenes* (14) and *K. aerogenes* (21), both *P. rettgeri* (9) and *P. aeruginosa* (15) were unaffected and *Pseudomonas* C12B was only partially responsive (11, 13). In *S. typhimurium*, arylsulfatase synthesis was virtually dependent upon tyramine when the organism was grown under a variety of conditions. Characteristically, arylsulfatase synthesis in bacteria is repressed by sulfate and cysteine and derepressed by methionine (3, 7, 8, 11, 12, 15, 17); however, with *S. typhimurium*, low levels of enzyme were found when the organism was grown with methionine in the absence of tyramine, and significant levels were detected with all three sulfur sources only in the presence of this compound.

In addition, all attempts to obtain significant levels of enzyme with different carbon sources in the absence of tyramine were uniformly negative. Curiously, there was a delayed synthesis of enzyme with all carbon sources tested when this compound was included in the growth medium. This suggested that initiation of arylsulfatase synthesis by tyramine was controlled by the presence of carbohydrate in the growth medium. These findings seem related to the work of Alper and Ames (4), which showed that, in *S. typhimurium*, synthesis of aromatic amino acid permeases (tyrosine, phenylalanine) was under cAMP control. It was suggested that each specific transport system was induced at a particular level of cAMP which, in turn, was reflected by the type of carbon compound present. Furthermore, albeit with *E. coli*, Peterkofsky et al. (23) reported that both phosphotransferase and non-phosphotransferase sugars were involved in the regulation of internal cAMP levels. The transport of both types of sugars differentially

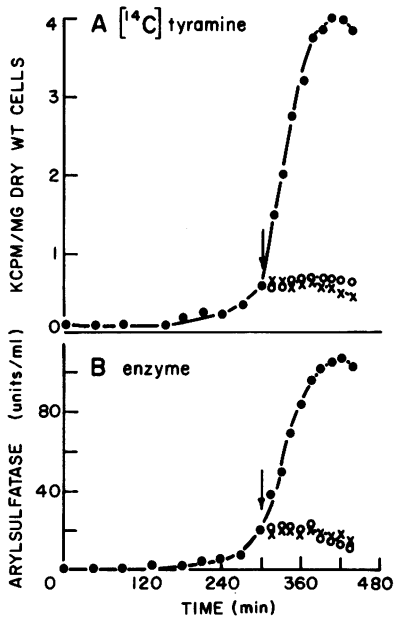

FIG. 6. *Effect of adding 3-O-methyl-D-glucopyranose to organisms synthesizing arylsulfatase in basal medium containing tyramine as the carbon source. Additions were made at the time indicated (↓). Symbols: ●, No additions; ○, 0.2% 3-O-methyl-D-glucopyranose; ×, 0.2% 3-O-methyl-D-glucopyranose plus 5 mM cAMP.*

inhibited adenylate cyclase activity and resulted in lowered intracellular cAMP concentrations. This would offer an explanation for the enhancement of tyramine transport and arylsulfatase synthesis by the addition of cAMP to *S. typhimurium* growing with glucose and glycerol. However, it seems unlikely that cAMP levels would be altered rapidly enough to account for the inhibitory response observed when glucose and glycerol were added to cells making enzyme. Also, the failure of cAMP to reverse this inhibition would seem inconsistent with the above explanation.

Recently, Saier et al. (27) presented evidence that, in *S. typhimurium* and *E. coli*, the permeases for inducers of catabolic enzyme systems were subject to allosteric regulation by the carbon source. Mutation in a particular permease rendered the system insensitive to regulation by all sugars, suggesting that repression of enzyme synthesis was due to inducer exclusion. In view of this information and the data presented in this paper, particularly the fact that 3-O-methyl-D-glucopyranose rapidly inhibited tyramine transport and subsequent arylsulfatase synthesis, it seems quite likely that the compound per se, and not metabolic products, was required to effect this inhibition. Therefore, it appears probable that the inhibition of tyramine transport may result from permease modification by the carbon source and that inducer exclusion may be the initial step in the regulation of arylsulfatase synthesis in this bacterium.

Several reports by the group studying arylsulfatase synthesis in *K. aerogenes* have repeatedly stressed the lack of arylsulfatase in *S. typhimurium* (2, 19, 32). Nonetheless, they have detected serologically an enzymatically inactive "latent arylsulfatase" in *S. typhimurium* (32) as well as an inactive, immunologically cross-reacting protein in an intergeneric hybrid of *E. coli–S. typhimurium* (19). It was suggested that synthesis of the "latent arylsulfatase" was governed by the same control mechanisms as arylsulfatase in *K. aerogenes* (19, 32). These findings are difficult to reconcile with our results.

In our view, a possible explanation for the lack of enzyme detection might reside with conditions employed by these workers (19). Their use of 0.5% carbon and 1 mM tyramine may have delayed enzyme synthesis beyond the period when enzyme measurements were made, and little or no arylsulfatase would, therefore, have been detected.

The contentions that the control of arylsulfatase synthesis has been conserved throughout the family *Enterobacteriaceae* (32) and that the latent arylsulfatase protein in *S. typhimurium* may have acquired some other enzyme activity (19) seem inconsistent with several aspects of our investigations. Firstly, we have shown that *S. typhimurium* synthesized an enzyme with properties comparable to those of arylsulfatases described in other gram-negative bacteria (6, 10, 22, 24). Secondly, in *K. aerogenes*, tyramine transport was not inhibited by the addition of

TABLE 1. *Influence of NH_4Cl and sulfur source on arylsulfatase synthesis in S. typhimurium*[a]

NH_4Cl concn (%)	Sulfur source	Arylsulfatase (U/ml)[b]	
		No tyramine	Tyramine
0.025	K_2SO_4 (1 mM)	4	15
0.05	K_2SO_4 (1 mM)	10	96
0.10	K_2SO_4 (1 mM)	10	96
0.30	K_2SO_4 (1 mM)	10	96
0.1	Cysteine (3 mM)	4	46
0.1	Methionine (3 mM)	3	47
0.1	K_2SO_4 (1 mM)	10	96

[a] The basal medium was modified by altering the initial concentration of NH_4Cl or sulfur source.

[b] Values reported represent the maximal level of enzyme attained within 8 h of growth.

glucose and xylose (3), whereas in *S. typhimurium* all carbon sources tested excluded this compound. Thirdly, derepression by methionine, ammonium repression, and catabolite repression as described in *K. aerogenes* (3, 21) was not found in *S. typhimurium*. Further, no active *S. typhimurium* arylsulfatase was found in intergeneric hybrids under conditions suitable for derepression of enzyme synthesis in *K. aerogenes* (19). In consideration of these points, the implication that the same elements control arylsulfatase synthesis in the two organisms seems untenable. Clearly, additional work must now be undertaken to elucidate the regulatory mechanisms governing arylsulfatase biosynthesis in *S. typhimurium* and other enteric bacteria.

ACKNOWLEDGMENTS

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