myocardium, a situation reflected in our enzyme fractions, or (2) the lability of the H_2 receptor to the procedures involved in obtaining the particulate enzyme preparations.

It is also possible that a more potent H_2-receptor antagonist than burimamide might allow a distinction between H_1 and H_2 antagonists at the subcellular level. Dousa and Code[13] have reported that stimulation by histamine of guinea pig gastric mucosal cyclase is blocked significantly by metiamide concentrations only 1% that of histamine. However, since they did not compare metiamide with an H_1 antihistamine in these studies, no definite conclusions about the uniqueness of action of metiamide *vs.* this enzyme preparation can be drawn.

The lack of potency of burimamide and its lack of specificity relative to mepyramine as a histamine receptor antagonist at the level of cardiac adenylate cyclase activity suggest that such enzyme complexes from myocardium may not be satisfactory molecular models for the H_2-receptor pharmacology of histamine in cardiac tissue.

Experimental Section

Enzyme Preparation. The preparation of guinea pig myocardial adenylate cyclase fractions has been previously described.[14] Briefly, the enzyme preparation is a once-washed particulate fraction derived from a 20% homogenate of ventricular tissue in $MgCl_2$ (1 m*M*)–glycylglycine (2 m*M*) buffer (pH 7.5), by centrifugation at 1000g for 15 min at 4°. Aliquots (0.5 ml) of the washed and resuspended pellets from the centrifugation were sealed in ampoules and stored under liquid nitrogen for future use. The results of Figures 4 and 6 were obtained with a particulate fraction, suspended in 10 m*M* Tris (pH 7.5),[12] that was used immediately after preparation.

Assay of Enzyme Activities. The assay for adenylate cyclase activity was essentially that reported by us previously.[14,15] [α-^{32}P]-ATP was the substrate and the isolation procedure for cyclic [^{32}P]-AMP included the use of cyclic [^{3}H]-AMP as a recovery standard, Dowex 50 chromatography, and $BaSO_4$ treatment. The basic assay system included 1.8 m*M* $MgCl_2$, 0.8 m*M* glycylglycine, 32 m*M* Tris (pH 7.8), 1.2 m*M* ATP, 5×10^6–10×10^6 cpm [α-^{32}P]-ATP, (50 μl) particulate enzyme fraction (125–225 μg of enzyme protein), 1 m*M* cyclic AMP, and test compound(s) in a total volume of 0.59 ml. ATPase activity was measured as previously outlined.[15] Cyclic AMP phosphodiesterase activity was determined by measuring the degradation of [^{3}H]-cyclic AMP in a cyclase assay mixture without ATP and in the absence and presence of 1 m*M* unlabeled cyclic AMP. Apparent cyclase activity was approximately linear with time in the absence and presence of histamine and tolazoline.

Materials. Isoproterenol (Schwartz/Mann) and histamine (Matheson Coleman and Bell) were obtained commercially. Tolazoline (Ciba-Geigy), mepyramine (Merck), and propranolol (Ayerst) were from the Squibb Institute chemical collection. Burimamide was a gift from Dr. J. W. Black (Smith Kline & French Laboratories, Ltd.). Other assay reagents were generally of analytical grade.

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Synthesis and Microbiological Activities of Some Monohalogenated Analogs of Tyrosine

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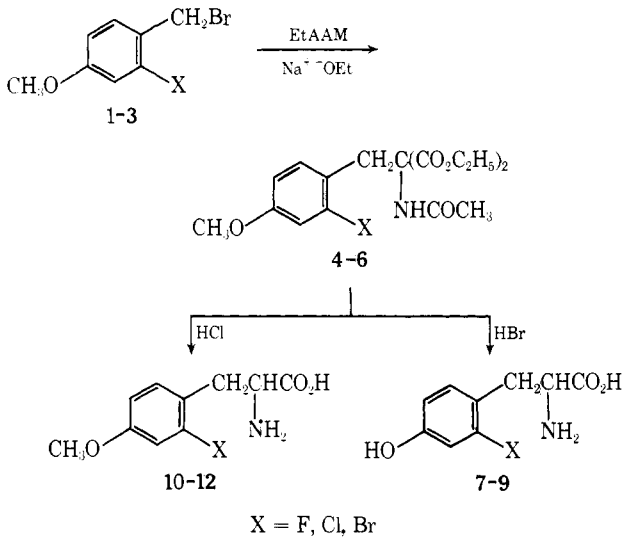
2-Chlorotyrosine and 2-bromotyrosine, as well as the previously reported 2-fluorotyrosine, were synthesized by hydrolysis of the condensation products from the appropriate benzyl bromide and ethyl acetamidomalonate and were compared with the corresponding 3-halotyrosines as growth inhibitors of *Escherichia coli* 9723, *Streptococcus faecalis* 8043, and *Lactobacillus plantarum* 8014. In contrast to the 2- and 3-fluorotyrosines which were equally effective as growth inhibitors, the 2-chloro- and 2-bromotyrosines were much more effective than the 3-chloro- and 3-bromotyrosines in inhibiting the growth of the three microorganisms. For each of the assay organisms, the growth inhibitions of all three 2-halotyrosines were reversed competitively in varying degrees by tyrosine.

Numerous studies on the preparation and biological activities of the various 3-monohalogenated and 3,5-dihalogenated tyrosines have been published. The general importance of these compounds results from their interrelationship to thyroxine biochemistry,[1] their isolation from natural sources,[2] and their abilities to act as metabolic inhibitors of biological systems.[3-5] On the other hand, only one of the four 2-halogenated tyrosines, 2-fluorotyrosine,[6] has been synthesized and virtually no study on its biological properties has appeared. Thus, the preparation of additional 2-halogenated analogs of tyrosine for microbiological studies was undertaken in the present study. Two new analogs of tyrosine, 2-chlorotyrosine and 2-bromotyrosine, were synthesized and studied together with 2-fluorotyrosine for growth-inhibiting properties in *Escherichia coli* 9273, *Streptococcus faecalis* 8043, and *Lactobacillus plantarum* 8014.

Chemistry. All three of the 2-halotyrosines **7–9** were

prepared by condensation of acetamidomalonic ester (EtAAM) with the appropriate 2-halo-4-methoxybenzyl bromides 1–3 followed by hydrolysis of the condensation products 4–6 with hydrobromic acid. Also, the structurally related 2-halo-4-methoxyphenylalanines 10–12 were obtained by hydrolysis of compounds 4–6 with hydrochloric acid.



Microbiological Studies. A summary of the inhibitory activities of the 2-halotyrosines and the 2-halo-4-methoxyphenylalanines in *E. coli* 9723, *S. faecalis* 8043, and *L. plantarum* 8014 is given in Table I. All three of the 2-halogenated tyrosines are effective growth inhibitors of each of the microorganisms studied. Moreover, they were uniformly more inhibitory to *L. plantarum* than the other two assay organisms. The order of increasing inhibitory activity varied for the individual 2-halotyrosines: F > Cl > Br in *E. coli*, F = Cl > Br in *S. faecalis*, and Cl > F > Br in *L. plantarum*. Although the 2-halo-3-methoxyphenylalanines 10–12 are completely inactive in *S. faecalis*, these compounds inhibited the growth of *E. coli* and *L. plantarum* but much less effectively than the 2-halotyrosines, with the exception of the 2-Br compound which was inactive in *E. coli*.

For comparative purposes, the inhibitory activities of 3-fluorotyrosine (15), 3-chloro-L-tyrosine (13), and 3-bromotyrosine (14) were determined under the same assay conditions and included in Table I. Since 3-fluorotyrosine was previously shown to antagonize tyrosine utilization competitively in several biological systems,[3,4,7] its activity served as a reference in the microbiological assays.

As indicated in Table I, both similarities and variations in toxicities of the 3-halotyrosines and 2-halotyrosines were observed. For example, the two isomeric fluorotyrosines possess about the same growth inhibitory activities in the three test organisms; however, the racemic 2-Cl analog is at least 10, 30, and 10,000 times more effective than the L form of the 3-Cl as a growth inhibitor of *E. coli*, *S. faecalis*, and *L. plantarum*, respectively. Furthermore, the 2-Br analog is active to some extent in all the microorganisms tested while the 3-Br is completely inactive up to a level of 200 μg/ml.

It follows from this comparative study of the activities of the 2-halo- and 3-halotyrosines that the type and position of the halogen have pronounced effects on the microbiological properties of the analogs. In the case of the fluorotyrosines, the position of the relatively small fluoro substituent has little if any effect on the inhibitory activity of the analog, whereas in the case of either the chloro or bromo substituent the 2 position is required for optimal inhibitory activity of the analog.

Since the 2-halo analogs as a group were more effective growth inhibitors than the 3-halo analogs, the effects of a 2 μg/ml supplement of tyrosine upon the amounts of the 2-halotyrosines necessary for inhibition of growth of *E. coli* 9763, *L. plantarum* 8014, and *S. faecalis* 8043 were determined, and the results are indicated in Table II. All three of the 2-halogenated tyrosines were competitively reversed by tyrosine, but there were some differences in the inhibition indices (ratio of concentration of 2-haloty-

Table I. Summary of Microbial Growth Inhibitions by Some Halotyrosines and Halo-4-methoxyphenylalanines

	Min inhibitory concn, μg/ml[a]		
	E. coli 9723[b]	*S. faecalis* 8043[b]	*L. plantarum* 8014[c]
2-Halotyrosines			
2-Cl (7)	2	6	0.02
2-Br (8)	20	20	0.2
2-F (9)	0.2	6	0.06
Halo-4-methoxyphenylalanines			
2-Cl (10)	200	>200	0.6
2-Br (11)	>200	>200	20
2-F (12)	60	>200	6
3-Halotyrosines			
3-Cl (13)	20	>200	>200
3-Br (13)	>200	>200	>200
3-F (15)	0.2	6	0.06

[a] Minimum inhibitory concentration required for complete inhibition of growth. [b] Growth media and assay conditions as described in the Experimental Section. [c] Growth media and assay conditions as described in the Experimental Section except that 5 μg/ml of phenylalanine was added to each assay tube.

Table II. Relative Inhibitory Activities of 2-Halotyrosines and Reversal by DL-Tyrosine in Various Microorganisms

2-Halotyrosine	Min inhibitory concn, μg/ml: Alone	2 μg/ml of DL-tyrosine	Inhibition index[a]
Escherichia coli 9723[b]			
2-Cl (7)	2	60	30
2-Br (8)	20	60	30
2-F (9)	0.2	60	30
Lactobacillus plantarum 8014[c]			
2-Cl (7)	0.02	60	30
2-Br (8)	0.2	200	100
2-F (9)	0.06	6	3
Streptococcus faecalis 8043[b]			
2-Cl (7)	6	60	30
2-Br (8)	20	200	100
2-F (9)	6	20	10

[a] The ratio of concentrations of 2-halotyrosine to that of tyrosine required for complete inhibition of growth. [b] Growth media and assay conditions as described in the Experimental Section. [c] Growth media and assay conditions as described in the Experimental Section except that 5 μg/ml of phenylalanine was added to each assay tube.

rosine to that of tyrosine necessary for complete inhibition of growth) for the three microorganisms.

Using *E. coli* 9723 as a model test organism, representative reversals of the growth inhibitions of the 2-halotyrosines by tyrosine are presented in Table III. Each of the 2-halo analogs competitively antagonizes the utilization of tyrosine for the growth of *E. coli* with an inhibition index of 30 over a broad range of increasing tyrosine concentrations. In contrast to these results, neither 6 μg/ml of phenylalanine nor 6 μg/ml of tryptophan reverses the inhibition of the 2-halotyrosines in this same microorganism.

This study represents not only the first report of the microbiological properties of 2-monohalogenated tyrosines but also demonstrates the marked effect that the 2-halo substituent has on the ability of the analog to act as a competitive antagonist of tyrosine.

Experimental Section

General. Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. Elemental analyses were performed by the M-H-W Laboratories, Garden City, Mich. Where analyses are indicated only by symbols of the elements, analytical results obtained by those elements were within ±0.4% of the theoretical values.

The following reaction procedures are described specifically for the chloro-substituted compounds; those indicated by reference to the particular table were prepared in a similar manner.

3-Halo-4-methylanisoles. The 2-chloro-,[8] 2-bromo-,[9] and 2-fluoro-substituted[6] 4-methylanisoles were prepared according to previously described procedures and their boiling points agreed with those reported in the literature.

2-Chloro-4-methoxybenzyl Bromide (Table IV, 1-3). A solution of 17.8 g (0.11 mol) of 3-chloro-4-methylanisole in 120 ml of anhydrous CCl_4 contained in a quartz flask was irradiated with a 100-W Hg vapor lamp as 24.2 g (0.14 mol) of *N*-bromosuccinimide and 2.4 g (0.01 mol) of benzoyl peroxide were added in five increments over 60 hr under reflux conditions. The succinimide was removed by filtration, and the red filtrate was passed through a column (3 × 15 cm) of Al_2O_3 (Alcoa F-20), which in turn was eluted with 400 ml of CCl_4. The effluent was evaporated *in vacuo* to an oil. Upon chilling the oily residue at −17° for several hours, the crystalline product separated. At this stage, a crystalline product could not be obtained for 3 and attempts to vacuum distill the oil resulted in appreciable decomposition. Thus, the oil of 3 was used directly in the acetamidomalonic ester synthesis. The crystals of 1 and 2 were collected by filtration, washed with cold *n*-hexane, and dried *in vacuo*. Additional crystalline product was recovered from the mother liquor and washings by reduction to dryness *in vacuo* followed by chilling the resulting oil at −17°. Analytical samples for 1 and 2 (physical constants, yields, and analyses, Table IV) were obtained by recrystallization from *n*-hexane-CCl_4 and vacuum sublimation, respectively.

Ethyl 2-Acetamido-2-(2′-chloro-4′-methoxybenzyl)malonate (Table V, 4-6). To a solution of 3.5 g (0.016 mol) of ethyl acetamidomalonate in 40 ml of absolute EtOH containing 0.38 g (0.07 mol) of Na was added slowly 3.5 g (0.015 mol) of 2-chloro-4-methoxybenzyl bromide with stirring at 25°. The reaction mixture was stirred for 1 hr during which time the product and NaBr separated from solution. The solid material was filtered, washed with cold H_2O to remove the NaBr, and dried *in vacuo* to yield the desired product. Recrystallization of the product from EtOH-Et_2O gave analytical samples (Table V).

2-Chloro-DL-tyrosine (Table VI, 7-9). A 1.0-g (0.0027 mol) sample of ethyl 2-acetamido-2-(2′-chloro-4′-methoxybenzyl)malonate (4) was hydrolyzed in the presence of 10 ml of refluxing 48% HBr for 24 hr. Upon cooling the acidic solution to 25°, the product separated as the HBr salt. The salt was removed by filtration and dried. A 0.53-g (0.0018) sample of the HBr salt was dissolved in a minimum amount of H_2O, and the pH of the solution was adjusted to 7 by the dropwise addition of 1 *N* NaOH solution to yield the free base. The free base was recovered and recrystallized from H_2O. Physical constants, yields, and analyses are given in Table VI.

2-Chloro-4-methoxy-DL-phenylalanine Hydrochloride (Table

Table III. Reversal of 2-Fluorotyrosine, 2-Chlorotyrosine, and 2-Bromotyrosine Inhibition by DL-Tyrosine in *Escherichia coli* 9723[a]

2-Halotyrosine	μg/ml	Absorbance readings,[b] DL-tyrosine, μg/ml 0	0.06	0.2	0.6	2	6	20
None		0.72	0.72	0.72	0.72	0.70	0.70	0.70
2-F (9)	0.06	0.70	0.72					
	0.2	0	0.70	0.71				
	0.6		0	0.71	0.72			
	2			0.02	0.68	0.70		
	6				0.10	0.40	0.68	
	20					0.21	0.54	
	60					0	0.40	
	200						0	
2-Cl (7)	0.6	0.72			0.73			
	2	0			0.68	0.72		
	6				0	0.40	0.68	
	20					0.18	0.59	0.70
	60					0	0	0.68
	200							0.40
	600							0
2-Br (8)	2	0.69			0.69			
	6	0.27			0.69	0.64	0.67	0.68
	20	0			0	0.12	0.51	0.54
	60					0	0.15	0.35
	200						0	0.25
	600							0

[a] Growth media and assay conditions as described in the Experimental Section. [b] A measure of culture turbidity in which absorbance readings of 0.70, 0.50, 0.30, 0.20, and 0.10 are equivalent to 0.37, 0.26, 0.15, 0.10 and 0.05 mg of dry weight of cells/ml of culture, respectively.

Table IV. 2-Halo-4-methoxybenzyl Bromides

Compd	X	Mp, °C	Yield, %	Formula	Analyses
1	Cl	53–55	24	C_8H_8BrClO	C, H
2	Br	59–60	15	$C_8H_8Br_2O$	C, H
3	F	[a]			

[a] Oil, not analyzed but used directly as described in the Experimental Section.

Table V. Ethyl 2-Acetamido-2-(2′-halo-4′-methoxybenzyl)malonates

Compd	X	Mp, °C	Yield, %	Formula	Analyses
4	Cl	128–129	80	$C_{17}H_{22}ClNO_6$	C, H, N
5	Br	135–136	57	$C_{17}H_{22}BrNO_6$	C, H, N
6	F	122–124[a]	36	$C_{17}H_{22}FNO_6$	

[a] Reported[6] mp 120–122°.

Table VI. 2-Halotyrosines

Compd	X	Mp, °C dec	Yield, %	Formula	Analyses
7	Cl	256–257	66	$C_9H_{10}ClNO_3 \cdot 0.5H_2O$	C, H, N
8	Br	255–256	51	$C_9H_{10}BrNO_3 \cdot H_2O$[a]	C, H, N
9	F	293–294[b]	68	$C_9H_{10}FNO_3$	C, H, N

[a] Also analyzed as HBr salt ($C_9H_{10}BrNO_3 \cdot HBr$) C, H, N. [b] Reported[6] mp 280–285° dec.

Table VII. 2-Halo-4-methoxyphenylalanines

Compd	X	Mp, °C dec	Yield, %	Formula	Analyses
10	Cl	245–247	81	$C_{10}H_{12}ClNO_3 \cdot HCl$	C, H, N
11	Br	249–250	88	$C_{10}H_{12}BrNO_3 \cdot HCl$	C, H, N
12	F	216–226[a]	56	$C_{10}H_{12}FNO_3$	C, H, N

[a] Reported[6] mp 217–221° dec.

VII, 10–12). Compound 4 (1.0 g, 0.0027 mol) was hydrolyzed with 20 ml of 6 *N* HCl at reflux for 6 hr. After the reaction mixture cooled to 25°, the salt which separated was collected by filtration. Recrystallization of the HCl salts of **10** and 11 gave analytical samples (Table VII); however, the salt of **12**, mp 186–189° dec, was converted to the free base by neutralization with concentrated NH_4OH.

3-Chloro-L-tyrosine (13). This compound was obtained from K & K Laboratories, Plainview, N.Y., and was recrystallized from aqueous ethanol and dried prior to its use in the microbiological assays.

3-Bromo-DL-tyrosine (14). This compound was prepared by adding an equivalent amount of a bromination solution (154 mg of $KBrO_3$ and 548 mg of KBr in 100 ml of H_2O) to a solution of 500 mg of DL-tyrosine in 0.5 *N* HCl. The solution was concentrated *in vacuo* to a few milliliters and let stand at 25°. The precipitated hydrohalide salts were collected by filtration and dried: yield 260 mg. In a separate experiment, the pH of an aqueous solution of 109 mg of hydrohalide salts was adjusted to 7 with 10% aqueous NaOH to yield 76 mg of the free base of 3-bromotyrosine, mp 245–246° (lit.[10] mp 247–248° *via* a different procedure).

3-Fluoro-DL-tyrosine (15). This compound was purchased from the Aldrich Chemical Co., Inc., Milwaukee, Wis., and was of sufficient purity to be used as the reference compound in the microbiological assays.

Microbiological Assays. For the growth inhibition and tyrosine reversal studies with *E. coli* (ATCC 9723) a previously described assay procedure[11] and inorganic salts–glucose medium[12] were used. For the growth assays with *S. faecalis* (ATCC 8043) and *L. plantarum* (ATCC 8014, *L. arabinosus* 17-5), the procedure and basal medium were the same as previously reported[13] except 100 μg/ml of calcium pantothenate was included in the vitamin supplement and both tyrosine and phenylalanine were omitted from the amino acid medium unless otherwise noted in the tables. The basal medium for *S. faecalis* was further modified by adding 20.0 μg/ml of glutamine under aseptic conditions.

The tyrosine analogs (10 mg) were dissolved in sterile H_2O (10 ml) at 25°. From these solutions, serial dilutions were made to the desired concentrations and added aseptically to the previously autoclaved assay tubes without being heated. After inoculation, the assay tubes with *E. coli* were incubated at 37° for 16 hr, and those with *S. faecalis* and *L. plantarum* were incubated at 30° for 24 and 30 hr, respectively.

In all assays the amount of growth was determined spectrophotometrically at 600 mμ with a Bausch & Lomb Spectronic 20 in terms of absorbance readings of the turbid culture medium against a blank of uninoculated medium set at zero absorbance. The milligrams of dry weight of cells were calculated from a standard curve of milligrams of cells plotted *vs.* absorbance readings. In all the assays, appropriate controls were run and the results of the minimum inhibitory concentrations of the various compounds tested were shown to be reproducible on repeating the assays at least 12 times.

Acknowledgments. The support of this work in part by research grants (R-285 and R-286) from the Robert A. Welch Foundation, Houston, Texas, and in part by a Cottrell College Science Grant from Research Corporation, New York, N.Y., is gratefully acknowledged.

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The Charge-Transfer Constant. A New Substituent Constant for Structure–Activity Relationships

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A new, versatile constant accounting for the π-complex formation ability of aromatic systems is proposed. The constant is derived from charge-transfer complex data of aromatic species (charge-transfer constant, C_T). The applicability of C_T in structure-reactivity relations is demonstrated. It is shown that the affinity of various inhibitors (32) and acetylcholinesterase is a function of C_T and π (Hansch hydrophobicity constant).

The analysis of the relation between structure and activity for a variety of drugs and toxicants has been much improved by two advances. One is the development of appropriate substituent constants which measure the effect of a substituent upon such factors as electronic character, size, or hydrophobicity. The second is the development by Hansch[1] of the technique of multiple regression analysis, by which it is possible to evaluate a situation which has, as one might have expected, turned out to be the most frequent case, *i.e.*, that the effectiveness of an agent is influenced by a number of factors, each of which is differently affected by a change in substitution.

Recently,[2,3] in studies on the relationship between structure and activity of carbamates as inhibitors of acetylcholinesterase, we have provided evidence that the formation of a charge-transfer complex (CTC) with the enzyme may be involved; at the moment the evidence is only indirect, because substantial quantities of highly pu-