Analogs of Tetrahydrofolic Acid XXXV

Hydrophobic Bonding to Dihydrofolic Reductase VII. Species Differences with Substituted 4,6-Diamino-1,2-dihydro-*s*-triazines

By B. R. BAKER* and BENG-THONG HO

Twenty-four 4,6-diamino-1,2-dihydro-*s*-triazines with varying substituents on the 1- and 2-positions have been compared as inhibitors of the dihydrofolic reductase from pigeon liver and from *E. coli* B. Since the 4,6-diamino-1,2-dihydro-*s*-triazine most probably binds to the enzyme in the same area as the pteridine ring of the substrate, dihydrofolate, little difference was seen in complexing of the 1, 2, 2-trimethyl derivative (VI); this result was anticipated since the active site of an enzyme cannot be varied appreciably and still have a functioning enzyme. In contrast, hydrophobic bonding to the enzymes by alkyl or aryl groups on the 1- and 2-positions of the inhibitors could be anticipated to show differences in binding between the two species, since it should be possible for the relatively nonfunctional hydrophobic region on the enzyme to be quite variable from species to species and still allow the enzyme to function. The biggest differences between the two species were observed when substituents were attached to the 1-phenyl group of 4,6-diamino-1-phenyl-2,2-dimethyl-1,2-dihydro-*s*-triazine (I) or larger groups than methyl were introduced on the 2-position. Differences in conformational requirements for hydrophobic bonding to the two enzymes are presented.

That alkyl or aryl groups attached to the 5-position of pyrimidines or the 1-position of 1,2-dihydro-*s*-triazines can give strong hydrophobic bonding to dihydrofolic reductase has recently been discovered (1); further studies (2) substantiated the proposal that the aryl groups were also bonded hydrophobically rather than by a charge transfer complex with the enzyme. Some of the conformational aspects of hydrophobic bonding to the dihydrofolic reductase from pigeon liver have also been investigated (3). The hydrophobic bonding region is most probably not in the area where dihydrofolate is complexed (4, 5); if it is adjacent to the 4-position or 8-position of dihydrofolate when the latter is complexed with dihydrofolic reductase (6), this hydrophobic region would therefore be in a nonfunctional part of the enzyme just adjacent to the active site. Furthermore, it might be expected that outside of the active site, one hydrophobic amino acid could be replaced by another hydrophobic amino acid without affecting the function of the active site, whereas amino acid replacement within the active site could be expected to be much more sensitive with respect to enzyme activity (7). *Ergo*, it can be anticipated that there should be species differences in the nonfunctional hydrophobic area presumed to be adjacent to the active site of dihydrofolic reductase. Some aspects of hydrophobic bonding to the dihydrofolic reductase from *E. coli* B and their relationship to the dihydrofolic reductase from pigeon liver is the subject of this paper. Furthermore, 2 previous studies—one from this laboratory (8) and one by Burchall and Hitchings (9, 10)—on the effect that substitution on the 5- and 6-positions of pyrimidines has on the comparative inhibition of dihydrofolic reductases from different sources can now be better envisioned when considered in the light of differences in hydrophobic bonding.

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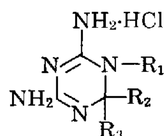
RESULTS AND DISCUSSION

Four discrete series of dihydro-*s*-triazines with variants at either the 1-position or 2-position were compared as inhibitors of the dihydrofolic reductase from pigeon liver and from *E. coli* B; the results are reported in Table I.

Series A.—*1-$C_6H_5(CH_2)_n$- Variants*.—The syntheses of the compounds in this series have been previously described (12). With both enzymes insertion of 1 methylene group (II) between the 1-aryl group and the *s*-triazine of I was detrimental to binding, being 10-fold with the *E. coli* B enzyme and thirtyfold with the pigeon liver enzyme; thus with both enzymes, when the aryl group is out of the plane of the triazine, a loss in binding occurs. By comparison of the 1-benzyl-*s*-triazine (II) with the 1-methyl-*s*-triazine (VI), it can be seen that the phenyl group of II gives little binding to the *E. coli* enzyme, but a 22-fold increment in binding to the pigeon liver enzyme, presumably due to the phenyl group, still remains. The next higher homolog, phenylethyl (III), can now have the phenyl ring nearly coplanar with the *s*-triazine ring; as a result, activity is better than benzyl (II) with both enzymes. On the *E. coli* B enzyme, the phenethyl (III) side-chain is just as effective as the phenyl side-chain (I); in contrast, phenethyl (III)

TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASES BY



Compd.	R_1	R_2	R_3	μM Concn. for 50% Inhibition[a] Pigeon Liver[b]	$E. coli$ B[c]	Pigeon Liver: E. coli B
I	$-C_6H_5$	CH_3	CH_3	0.11[d]	3.0	1/27
II	$-CH_2C_6H_5$	CH_3	CH_3	3.3[d]	34	1/10
III	$-(CH_2)_2C_6H_5$	CH_3	CH_3	0.71[d]	3.8	1/5.3
IV	$-(CH_2)_3C_6H_5$	CH_3	CH_3	0.028[d]	0.063	1/2.2
V	$-(CH_2)_4C_6H_5$	CH_3	CH_3	0.041[d]	0.21	1/5.1
VI	$-CH_3$	CH_3	CH_3	74[e]	48	1.5
VII	$-C_2H_5$	CH_3	CH_3	200[e]	62	3.5
VIII	$-C_3H_7$-n	CH_3	CH_3	11[e]	4.7	2.3
IX	$-C_4H_9$-n	CH_3	CH_3	0.36[e]	2.1	1/5.8
X	$-C_5H_{11}$-i	CH_3	CH_3	0.058[e]	0.69	1/12
XI	$-C_6H_{13}$-n	CH_3	CH_3	0.32[e]	0.92	1/2.9
XII	$-C_8H_{17}$-n	CH_3	CH_3	0.14[e]	0.27	1/1.9
XIII	$-C_6H_4-C_6H_5$-p	CH_3	CH_3	160[f]	5.8	28
XIV	$-C_6H_4-C_6H_5$-m	CH_3	CH_3	1.3[f]	1.0	1.3
XV	$-C_6H_4-(C_4H_9$-$n)$-p	CH_3	CH_3	0.064[f]	7.2	1/110
XVI	9-fluorenone-2-yl	CH_3	CH_3	85[d]	1.5	57
XVII	$-C_6H_4C(=O)C_6H_5$-m	CH_3	CH_3	1.1[d]	0.61	1.8
XVIII	$-C_6H_4Cl$-m	CH_3	CH_3	0.0085[g]	0.60[g]	1/71
XIX	$-C_6H_4CH_2C_6H_5$-p	CH_3	CH_3	0.062[f]	4.8	1/77
XX	$-C_6H_4CH_2C_6H_5$-m	CH_3	CH_3	0.019[d]	0.28	1/15
XXI	$-C_6H_4CH_2CH_2C_6H_5$-m	CH_3	CH_3	0.024[d]	0.092	1/3.5
XXII	$-(CH_2)_4C_6H_5$[h]	p-AcNHC$_6$H$_4$—	H	0.62[d]	0.41	1.5
XXIII	$-C_6H_4Cl$-m	p-AcNHC$_6$H$_4$—	H	190[d]	180	1.0
XXIV	$-C_2H_5$	$-C_6H_5$	H	15,000[d]	310	50

[a] The technical assistance of Maureen Baker and Karen Smith with these assays is acknowledged. [b] A 45–90% saturated ammonium sulfate fraction prepared from pigeon liver acetone powder and assayed with 6 μM dihydrofolate and 12 μM TPNH as previously described (11) at pH 7.4 in Tris buffer. [c] *E. coli* B cell walls were broken with a French pressure cell at 20,000 p.s.i. A 45–90% saturated ammonium sulfate fraction was prepared as previously described (11), then assayed with 6 μM dihydrofolate and 30 μM TPNH. [d] Data from *Reference 12*. [e] Data from *Reference 1*. [f] Data from *Reference 2*. [g] Data from *Reference 8*. [h] Picrate salt.

is 6.5-fold less effective than phenyl (I) with the pigeon liver enzyme.

Maximum effectiveness against both enzymes is shown with the phenylpropyl side-chain (IV), with phenylbutyl (V) being 1.5–3 times less effective. However, the increment between phenyl (I) and phenylpropyl (III) is much larger (48-fold) with the *E. coli* enzyme than with the pigeon liver enzyme (fourfold); most of this difference is due to the fact that the 1-phenyl (I) binds to the pigeon liver enzyme 27-fold better than the *E. coli* B enzyme, possible reasons for which are discussed later. Furthermore, the greatest specificity toward the pigeon liver enzyme (27-fold) is shown by the 1-phenyl side-chain (I), but none of the compounds in this series are more effective on the *E. coli* B enzyme than the pigeon liver enzyme.

Series B.—*1-Alkyl Variants.*—The synthesis of these compounds has been previously described (1). With straight-chain alkyl substituents maximum hydrophobic bonding with the pigeon liver enzyme occurred in n-butyl (IX) with n-hexyl (XI) being equal and n-octyl (XII) perhaps twofold better; in contrast, with the *E. coli* B enzyme, n-hexyl (XI) was 2.3-fold better than n-butyl (IX) and n-octyl (XII) was 3.4-fold better than n-hexyl (XI). Extension of the series would be required to reveal whether maximum straight-chain hydrophobic bonding to the *E. coli* B enzyme had occurred at n-octyl.

Branching of the n-butyl group (IX) to isoamyl (X) gave a sixfold increment in binding on the pigeon liver enzyme, and X was the most effective alkyl group found for the enzyme from this source (1, 3); the isoamyl group (X) gave a threefold increment in binding over the n-butyl group (IX) with the *E. coli* enzyme, but—in contrast to the pigeon liver enzyme—the isoamyl group (X) was still less effective than the n-octyl group (XII). Whether chain-branching farther out the chain than the C-3 branch of isoamyl (X) would give a still better inhibitor of the *E. coli* B enzyme is as yet unknown.

It was previously noted (1) that the increment in binding between methyl (VI) and n-butyl (IX) to the pigeon liver enzyme was 200-fold; this is well within the 1000-fold (10-fold per methylene) that is theoretically possible. However, it was pointed out that there was a twentyfold increment in binding in proceeding from ethyl (VI) to n-propyl (VIII) and a thirtyfold increment in n-propyl (VIII) to n-butyl (IX), both of which are above the 10-fold increments theoretically possible. The fact that methyl to butyl gave only a 200-fold increment—which is within the theoretical 1000-fold

increment in binding for 3 methylene groups—was rationalized by assuming a conformational change in the enzyme; if this conformational change in the enzyme required no more than 900 cal./mole, then this 900 cal./mole could be supplied by the fivefold difference between the 200-fold increment observed in proceeding from methyl to butyl and the 1000-fold increment theoretically possible. Thus, the greater than 10-fold increment in binding between *n*-propyl and ethyl or *n*-butyl and *n*-propyl is still energetically possible including the energy required for a conformational change in the pigeon liver enzyme. With the *E. coli* B enzyme only the change between ethyl and *n*-propyl gave greater than a 10-fold increment (13-fold) which indicates that little or no conformational change in this enzyme need take place for maximum hydrophobic binding; it should be further noted that the maximum increment in hydrophobic bonding by straight-chain alkyl groups with the *E. coli* enzyme is only 180-fold (between methyl and *n*-octyl) whereas the pigeon liver enzyme has a maximum increment of 530-fold between methyl and *n*-octyl groups.

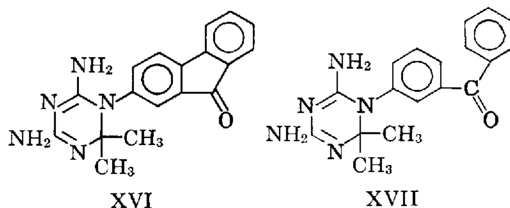
In this series, the isoamyl-*s*-triazine (X) shows the most specificity toward the pigeon liver enzyme, being 12-fold more effective than on the *E. coli* B enzyme; the most specific compound toward the *E. coli* B enzyme is the ethyl-*s*-triazine (VII) which is 3.5-fold more effective than on the pigeon liver enzyme.

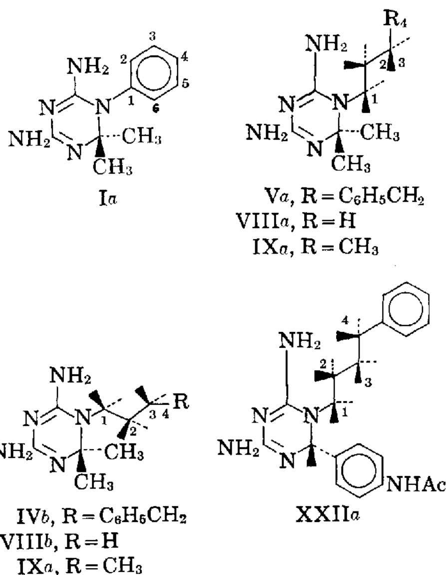
Series C.—*1-Phenyl with m- and p-Substituents.*—The synthesis of these compounds and their inhibition on pigeon liver dihydrofolic reductase have been previously described (2, 12). In this series is seen the largest spread in specificity between the two enzymes in both directions. The *E. coli* B enzyme is relatively insensitive to *m*- or *p*-substituents, the maximum variation being between 7.2 μM for the *p*-(*n*-butyl)-substituent (XV) to 0.6 μM for the *m*-benzoyl (XVII) and *m*-chloro (XVIII) substituents, a 12-fold spread; in contrast, the pigeon liver enzyme is extremely sensitive to substitution, varying from 0.0085 μM for the *m*-chlorophenyl group (XVIII) to 160 μM for the *p*-biphenyl group (XIII) at the 1-position of the *s*-triazine, a 19,000-fold spread. As a result the *p*-(*n*-butyl)-phenyl-*s*-triazine (XV) is 110-fold more effective on the pigeon liver enzyme than the *E. coli* B enzyme; similarly, the *m*-chlorophenyl-*s*-triazine (XVIII) and the *p*-(benzyl)phenyl-*s*-triazine (XIX) are 71 and 77-fold, respectively, more effective on the pigeon liver enzyme. In contrast, the *p*-biphenyl-*s*-triazine (XIII) and the 9-fluorenon-2-yl-*s*-triazine (XVI) are 28 and 57-fold more effective, respectively, on the *E. coli* B enzyme. Note that the benzoyl group of XVII causes a 10-fold loss in binding to the pigeon liver enzyme, but a fivefold gain in binding to the *E. coli* B enzyme. When the *m*-benzoylphenyl group of XVII is cyclized to the 9-fluorenon-2-yl group as in XVI, only a 2.5-fold loss in binding occurs in the *E. coli* B enzyme, but a further 77-fold loss in binding to the pigeon liver enzyme occurs; thus, the pigeon liver enzyme cannot tolerate a large flat substituent at the 1-position, but the *E. coli* B enzyme can. Reduction of the ketone of XVII to give the *m*-benzyl derivative (XX) causes little change with the *E. coli* B enzyme, but 46-fold better inhibition is seen with the pigeon liver enzyme.

The most active and one of the most specific compounds for the pigeon liver enzyme in this series is *m*-chlorophenyl-*s*-triazine (XVIII) at 0.0085 μM. The most active compound in this series for the *E. coli* B enzyme is the *m*-phenethylphenyl-*s*-triazine (XXI) at 0.092 μM, but this compound has no specificity toward the *E. coli* B enzyme since XXI is even more effective on the pigeon liver enzyme.

Series D.—*Variants at the 2-Position.*—The synthesis of these compounds and their evaluation on pigeon liver dihydrofolic reductase have been previously described (12). Structural change at the 2-position by introduction of larger groups has a much more detrimental effect on binding to the pigeon liver enzyme than the *E. coli* B enzyme. Note that replacement of the 2,2-dimethyl group of the 1-phenylbutyl-*s*-triazine (V) by 2-(*p*-acetamidophenyl) (XXII) reduces binding to the pigeon liver enzyme by a factor of 15-fold, but reduces binding to the *E. coli* enzyme by only a factor of twofold. Similarly, the same 2-substituent exchanged on the 1-(*m*-chlorophenyl)-*s*-triazine (XVIII) to give XXIII causes a 19,000-fold loss in binding to the pigeon liver enzyme, but only an 850-fold loss to the *E. coli* B enzyme; it should be noted that this structural change giving a 1,2-diaryl-*s*-triazine reduces coplanarity of at least one phenyl and possibly both phenyls with the *s*-triazine ring (13). When the 2, 2-dimethyl group of the 1-ethyl-*s*-triazine (VII) is replaced by phenyl to give XXIV, it results in only a fivefold reduction in binding to the *E. coli* B enzyme, but a 68-fold reduction in binding to the pigeon liver enzyme occurs. A study of other 2-substituents with IV and V could well lead to higher affinity to the *E. coli* B enzyme than the pigeon liver enzyme since the latter is more sensitive to structural change at this 2-position; note that XXIV, although it is a poor inhibitor, is fiftyfold more effective on the *E. coli* B enzyme than the pigeon liver enzyme and that no compound in this series shows the converse order of affinity.

Relative Topography of Hydrophobic Bonding to the Two Enzymes.—The difference in binding to the two enzymes can give considerable insight into the differences in topography of the hydrophobic regions of the two enzymes. It is well established that the phenyl group of I*a* must be nearly coplanar with the triazine ring for maximum binding (13) to the enzyme since introduction of an *o*-chloro group causes a great reduction in the affinity of the inhibitor to both enzymes due to the restricted rotation of the 1-phenyl-*s*-triazine system imposed by the *o*-chloro group (8, 12). Since there is strong experimental support for the 1-phenyl group being hydrophobically bonded to the enzyme (2), it is a reasonable assumption that the 1-phenyl group of I*a* and a 1-alkyl group of VIII*a* are bonded in the same





region. The alkyl group can then be staggered in the plane of the triazine either away from the 2-position as in VIII*a* or toward the 2-position as in VIII*b*. Since XXII and XXIV can still bind reasonably well to both enzymes, a downward-stagger of the VIII*b* type is not possible due to the 2-phenyl group of XXII occupying the same space as the C_2-methylene group of the side chain. With the upward-staggered conformation, there is no steric interaction between the C_2-methylene group and the 2-phenyl, but there is now a possible steric interaction between the C_1-methylene group and the 2-phenyl group; this C_1-methylene steric interaction does not occur if the 2-phenyl group is in a plane perpendicular to the triazine, but is strong if the plane of the phenyl group is moved 90°. It follows that the *E. coli* B enzyme has more tolerance for the 2-phenyl group approaching a plane perpendicular to the triazine than the pigeon liver enzyme, since little activity is lost in the *E. coli* B enzyme when VII is converted to XXIV or V is converted to XXII; the large reduction in affinity to the pigeon liver enzyme indicates that this enzyme cannot tolerate the 2-phenyl group approaching a plane perpendicular to the *s*-triazine ring.

Note that the *n*-propyl-*s*-triazine (VIII) is nearly as effective as the 1-phenyl-*s*-triazine (I) on the *E. coli* B enzyme, but that I is 100-fold more effective than VIII on the pigeon liver enzyme. Models clearly indicate that C_1, C_2, and C_3 of the phenyl group exactly overlap the C_1, C_2, and C_3 groups of an alkyl side chain when they have conformations I*a* and VIII*a*, respectively. Since the 1-propyl-*s*-triazine (VIII*a*) binds almost as well as the 1-phenyl-*s*-triazine (I*a*) to the *E. coli* enzyme, it is reasonable to propose that most of the phenyl bonding of I*a* occurs through the C_1, C_2, and C_3 carbons; the remaining 1.6-fold difference between I*a* and VIII*a* might be due to slight additional bonding by the C_4 or C_6 carbons of the phenyl or the C_1, C_2, and C_3 carbons of the phenyl of I*a* may hydrophobically bond a little better than the corresponding carbons of VIII*a;* this 1.6-fold difference, however, is practically negligible.

The 100-fold better binding of the 1-phenyl of I*a* than the 1-propyl of VIII*a* to the pigeon liver enzyme suggests that at least 2 more carbons of the benzene ring—in addition to C_1, C_2, and C_3—are complexed hydrophobically to this enzyme; one of these carbons is most probably C_4 since the isoamyl group (X) with its skewed C_4 and C_4' carbons has been shown to have both of these carbons hydrophobically bonded (3).

Both the *n*-octyl (XII) and the phenylbutyl-*s*-triazine (IV*a*) are better—and about equally effective—inhibitors of the *E. coli* B enzyme than the 1-phenyl-*s*-triazine (I*a*) by a factor of 11-to 14-fold. Furthermore, XII and IV*a* are about 10-fold better inhibitors than the *n*-butyl-*s*-triazine (IX*a*); since the terminal four carbons of XII can only increase affinity to the enzyme by hydrophobic bonding of one or more of these terminal carbons, it follows that the phenyl group of IV*a* does likewise. In contrast, the pigeon liver enzyme has only a twofold greater affinity for the octyl group of XII than the butyl group of IX*a*, but the phenylbutyl group of IV*a* is complexed eightfold better than the butyl group of IX*a;* it follows that the additional hydrophobic bonding to the pigeon liver enzyme past the *n*-butyl group requires the relatively flat interaction that can occur with a benzene ring, but the *E. coli* B enzyme can bind either a flat or staggered group in this area. The greater tolerance to type of group attached to the 1-phenyl substituent by the *E. coli* B enzyme has also been pointed out under *Series C*.

Of further interest is that there is less than a twofold difference in binding to the two enzymes with the 1-methyl-*s*-triazine (VI), but there is a 12-fold difference with the *i*-amyl-*s*-triazine (X) and even larger differences in both directions with XV and XVI. (See *Series C*.) Since it is highly probable that the 4,6-diamino-*s*-triazine moiety binds within the normal complexing region for the pteridine ring of the substrate, dihydrofolate, and since the active site of an enzyme has a considerable limitation on structural change which will still allow the enzyme to be operable, little difference should be seen in binding of the 4,6-diamino-*s*-triazine moiety to the enzyme. In contrast, large differences in hydrophobic bonding were anticipated and found since this is a nonspecific part of the enzyme which could readily vary from species to species and still leave an operable enzyme.

Further examples of species differences in the ability of dihydrofolic reductase to bind 2,4-diamino-heterocycles that have varying hydrophobic groups have been collated by Burchall and Hitchings (9); their observations fit into the differences in hydrophobic bonding that are described here. They also noted that the biggest species differences existed when substituents on the 1-phenyl group or 2-position of the *s*-triazine were varied.

Although differences in hydrophobic bonding to the dihydrofolic reductase between invading organisms such as bacteria and protozoa on the one hand, and mammalian enzymes on the other hand, are sufficiently large to enjoy a chemotherapeutic advantage with reversible inhibitors *in vivo* (9), such differences between a cancer cell and the host are most probably too small to have any chemotherapeutic advantage with reversible inhibitors of the 2,4-diamino-heterocycle type. By utilizing both hydrophobic bonding to dihydrofolic reductase

and the bridge principle of specificity with active-site-directed irreversible inhibitors (14), it should be possible to magnify immensely any small difference between the hydrophobic areas of the tumor tissue and host dihydrofolic reductases; this small difference would be unusable or even undetectable with reversible inhibitors. Since active-site-directed irreversible inhibitors of the dihydrofolic reductase from pigeon liver have been found which utilize the hydrophobic bonding region (15, 16), only a small difference in the hydrophobic region of a tumor tissue dihydrofolic reductase would be sufficient for high specificity—such as a change of a valine for a leucine or a slightly different conformation in the hydrophobic region caused by a single amino acid exchange in another region. Such a small difference could be exploited by attaching a bridging moiety to the hydrophobic bonding moiety of an inhibitor so that the subsequent attack by the bridging group to form a covalent bond to a nucleophilic site on the enzyme is subject to proper juxtapositioning by the hydrophobic area on the enzyme. Such studies are currently being pursued.

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Effect of Certain Drugs on Perfused Human Placenta VI

Serotonin Antagonists

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The antiserotonin action of several compounds was investigated in the vasculature of the isolated perfused human placenta. Average onset, duration of action, and per cent decrease at maximal antagonism were used to discern the antiserotonin capability of the smallest effective dose of each compound necessary to antagonize the pressor action of serotonin. Their ability to antagonize the vasoconstrictor effect of serotonin, in decreasing order of effectiveness, was: cyproheptadine, LSD, diphenhydramine, chlorpromazine, promethazine, promazine, and dibenamine. Cyproheptadine, which had a relatively short duration of action, caused the greatest decrease to the pressor action of serotonin, while chlorpromazine and diphenhydramine exhibited the longest duration of action. The mechanisms by which these agents antagonized the vasopressor effect of serotonin are attributed to a blockade of α-adrenergic receptors, competition for specific receptor sites, and/or direct negative musculotropic action. It is suggested that the human placenta, rather than tissues of other species, may serve as the organ of choice to evaluate the potential effectiveness of serotonin antagonists useful in therapeutics.

Although the vasoconstrictor property of serotonin has been known since 1869 (1), the interplay between its physiologic and pathologic functions, as well as the elucidation of its pharmacologic mechanisms, awaited the development not only of reliable assays for its estimation in biologic tissues, but also techniques for determining the potency of agents specifically antagonistic to it.

Many compounds have been shown to antagonize the stimulant effect of serotonin on smooth muscle, and among the several organs used to demonstrate this phenomenon are the gastric fundus of the rat (2), estrogen-primed rat uterus (3), isolated rabbit ear (4), sheep artery rings (5), and the guinea pig ileum (6). The effect of serotonin on diuresis (7) and blood pressure (8) in the intact animal also has been used to show the antiserotonin effect of these agents. While these techniques are generally sensitive to minute doses of serotonin, and though the results are usually reproducible in the particular procedure

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