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Differences in the stereospecificity of closely related compounds; a reinvestigation of the enantiomers of procyclidine, benzhexol and their metho- and etho-salts

R. B. BARLOW

Department of Pharmacology, University of Edinburgh, 1 George Square, Edinburgh, U.K.

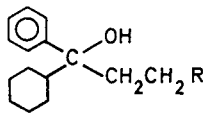
The findings of Duffin & Green (1955) that there are very large differences in the stereospecificity of some closely related phenylcyclohexylhydroxypropyl compounds have been confirmed, and it has been shown that this cannot be ascribed to any errors attached to the methods for assessing biological activity, or to inadequate resolution of some of the compounds. Measurement of the affinity constants of the compounds for the postganglionic acetylcholine receptors of the guinea-pig ileum showed that the (+)-and (−)-isomers of benzhexol differ only 5·5-fold in affinity whereas the (+)- and (−)-isomers of procyclidine differ at least 375-fold. This big variation in stereospecificity indicates that changes in one part of the molecule markedly affect the binding of the rest of the molecule and the effects are different in the different enantiomers. It is not possible to interpret the difference between the affinity of the isomers simply in terms of the fit, or failure to fit, of one group, such as the hydroxyl, attached to the asymmetric centre. In the five pairs of compounds tested, the stereospecificity was greatest in the compounds with lowest affinity, which is the reverse of what would be predicted from Pfeiffer's rule.

The difference between the biological activities of optical isomers has long interested pharmacologists and is one of the most important reasons for believing in the existence of "receptors". Because enantiomers differ only in the arrangement of groups about an asymmetric centre, it is assumed that the difference between their biological activities (the stereospecificity of the pair) yields information about the asymmetry of the receptor. For compounds which are agonists, such as the enantiomeric forms of noradrenaline, adrenaline and isoprenaline, the differences in activity depend upon differences in ability to activate receptors, as well as upon differences in affinity for the receptors; the stereospecificity of such agonists cannot, therefore, be used satisfactorily to analyse receptor structure. With antagonists, however, the stereospecificity is due only to differences in the affinity of the two forms.

Accordingly, the high stereospecificity of the isomers of phenylcyclohexylglycollylcholine as antagonists of furfuryl trimethylammonium on rat intestine (Ellenbroek, Nivard & others, 1965) can be taken to indicate the existence in the "muscarine-sensitive" receptor of groups capable of interacting with cyclohexyl, phenyl and hydroxyl groups as well as with the charged onium group in the antagonists. The discovery by Inch, Ley & Rich (1968) that the more active (−)-isomer has the *R* configuration then makes it possible to indicate how these groups may be arranged in the receptor (see, for instance, van Rossum, 1968).

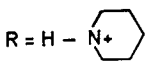
Measurement of the stereospecificity of isomeric antagonists is undoubtedly a valuable method of investigating receptor structure, particularly as there is as yet no means of studying this directly with isolated receptors, but it seems likely that there are limits to the usefulness of results obtained with only one pair of isomers. From measurements of the affinity of many series of antagonists at the postganglionic acetylcholine receptors of the guinea-pig ileum, Abramson, Barlow & others (1969) obtained evidence that the binding of one part of an antagonist at the receptor may be considerably affected by changes in the structure of other parts of the molecule. Changes in chemical structure at points distant from the asymmetric centre may therefore have profound effects on stereospecificity.

The results by Duffin & Green (1955), who measured the atropine-like activity of enantiomeric pairs of phenylcyclohexylhydroxypropyl compounds, I, showed this to

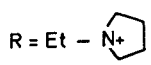


I

be so. The stereospecificity varied, depending on the nature of the group R at the other end of the molecule from the asymmetric centre. The size of the variation was particularly striking. In tests on the guinea-pig ileum the stereospecific index for the piperidino-compounds, with



was 9·8 whereas for the ethylpyrrolidinium compounds, with



it was 290. Because of the important implication of these results it seemed desirable to check them. Drs Duffin and Green very kindly provided samples of the compounds and tests have been made of their biological activity on the guinea-pig isolated ileum and on the optical purity of some of them.

METHODS

Compounds. The substances tested had the basic structure I with R = the groups shown in Table 1.

Biological activity. The affinity constants of the compounds for the "muscarine-sensitive" postganglionic acetylcholine receptors of the guinea-pig ileum at 37° were measured by the method of Abramson & others (1969). Carbachol was the agonist. In most experiments the dose-ratio (Gaddum, Hameed & others, 1955) obtained when the antagonist had come into equilibrium with the tissues was between 20 and 100 but if there was enough material, tests were also made with higher concentrations, which produced dose-ratios between 100 and 1000. The results obtained with high concentrations gave values of the affinity constant similar to those obtained with lower concentrations, which is consistent with competitive antagonism. For example, in an experiment with the (−)-isomer of procyclidine, 364C52, the value of K was $1{\cdot}58 \times 10^8$ for a dose-ratio of 32·6 and $1{\cdot}88 \times 10^8$ for a dose-ratio of 380.

Duffin & Green (1955) measured the atropine-like activity of the compounds on the guinea-pig isolated ileum at 38° by comparing the concentrations of the compounds and of atropine which reduced the size of the response to a standard dose of acetylcholine by one-half (White, Green & Hudson, 1951; Green, 1953).

Optical purity. The rotations of the isomers of procyclidine (363C52 and 364C52), of benzhexol (247C53 and 248C53), and of the (−)-forms of their metho-salts (427C52 and 250C53), were measured with a Bellingham and Stanley Model B Spectropolarimeter. The cell had a 5 cm light-path and readings were taken at 17–20° with solutions in water and in chloroform which were usually 2×10^{-2}M, In some instances it was necessary to use more dilute solutions because of the shortage of material. The wavelengths selected were 546, 320, 300, 290 and 280 nm, but with solutions in chloroform the absorption by the solvent made it impossible to obtain readings below 290 nm and the absorption by the iodide ion similarly limited the readings that could be obtained with the methiodides dissolved in water.

All measurements were made relative to air. Mean values for the rotation at each wavelength were calculated, together with estimates of the standard error, based on the variance of the values for the solutions and the values for the solvent, but it is probable that these are underestimates. The results obtained with 247C53 and 248C53, for instance, indicate that the rotation of the (+)-isomer is significantly less than that of the (−)-isomer ($P < 0{\cdot}05$) at 546 and 320 nm and significantly greater at 290 nm, even though the same solutions were used.

There was a steady increase in the size of rotation with increasing wavelength. Dr. J. C. P. Schwarz and Mr. F. Rutherford kindly measured the rotations of 427C52 and 364C52 with a continuously scanning instrument (Bellingham and Stanley Polarmatic 62) down to 240 nm and observed only small Cotton effects, insufficient to alter the sign of rotation, in the regions corresponding to the absorption maxima (262, 257 and 251 nm). The signs of rotation of the hydrochlorides of the tertiary bases in water, however, were the opposite of those for solutions in chloroform and the size of rotation was smaller. The compound 364C52, designated the (−)-enantiomer of procyclidine because of its rotation in chloroform, was dextrorotatory in water at all the wavelengths studied. The quaternary salts, on the other hand, had the same sign of rotation in water as they had in chloroform, though the size of rotation was again smaller in water.

RESULTS

Table 1 shows the logarithms of the affinity constants for the postganglionic acetylcholine receptors of the guinea-pig ileum at 37°. Mean values are given together with the standard error and the number of estimates. Each estimate was made on a fresh piece of ileum. The three compounds which were not tested in high concentrations to check for competition are indicated. The stereospecific index (difference between the activity of the enantiomers) can be compared with the values obtained by Duffin and Green, which are shown in parentheses. The activity of the compounds relative to atropine has been calculated assuming a value of log K for atropine of 9·0 and are molar; the values obtained by Duffin and Green were calculated according to weight.

Most of the results do not differ from the original estimates by more than a factor of two. The biggest discrepancy is with the pyrrolidino-compounds, 363C52 and 364C52, where the stereospecific index is much higher than that obtained previously.

Table 1. *Logarithms of the affinity constants for postganglionic acetylcholine receptors of the guinea-pig ileum at 37° together with the stereospecific index and activity relative to atropine.*

		$\alpha_{546}^{20°}$	log K ± s.e. (*n* results)	Stereospecific index	Activity relative to atropine
Enantiomers of procyclidine					
 HCl	(−) 364C52	− 30	8·266 ± 0·014 (10)	375 (49)	0·18‡ (0·10)
	(+) 363C52	+ 30	5·692 ± 0·043 (6)†		0·00049 (0·002)
	(±)		7·945 ± 0·013 (4)		
Enantiomers of tricyclamol					
MeI	(−) 427C52	− 25	8·702 ± 0·027 (7)	87 (160)	0·50 (1·6)
	(+) 428C52	+ 25	6·732 ± 0·026 (6)		0·0054 (0·01)
EtI	(−) 429C52	− 30	8·684 ± 0·018 (8)	226 (290)	0·48 (1·0)
	(+) 430C52	+ 30	6·330 ± 0·035 (7)†		0·0021 (0·0034)
Enantiomers of benzhexol					
 HCl	(−) 248C53	− 30	8·751 ± 0·015 (7)	5·5 (9·8)	0·56 (0·71)
	(+) 247C53	+ 31	8·008 ± 0·033 (9)		0·10 (0·075)
MeI	(−) 250C53	− 22	9·175 ± 0·050 (20)	86·3 (48)	1·50 (0·86)
	(+) 249C53	+ 23	7·239 ± 0·037 (8)†		0·017 (0·018)
EtI	(−) 375C53	− 18	9·729 ± 0·030 (8)		5·36

* The values of the rotations are those of Duffin & Green (1955). The enantiomer of 375C53 had a rotation of +21 but none was left for testing.
† Not tested in concentrations which produced dose-ratios >100.
‡ Molar and assuming log K for atropine = 9·0; values in parentheses from Duffin & Green are on a weight basis.

The high value, however, is confirmed by tests made with a racemic solution, obtained by mixing equal amounts of equimolar solutions of the two enantiomers. The value of log K for this was less than that for the more active (−)-isomer by 0·321, which is not significantly different from log 2.

The low stereospecificity of the piperidino-compounds, 247C53 and 248C53, and the high stereospecificity of the pyrrolidino-compounds, 363C52 and 364C52, has therefore been confirmed. It is highly unlikely that the logarithms of the stereospecific index are incorrect by as much as 0·1, i.e that the stereospecific index is incorrect by more than a factor of 1·3. It is next necessary to consider whether the low stereospecificity of the piperidino compound might be due to inadequate resolution of the isomers.

Suppose that x mol of the weaker isomer produce the same biological effect as 1 mol of the stronger, when both are absolutely pure. If the weaker isomer is only partly resolved, however, and contains a fraction y, it will also contain a fraction (1 − y) of the stronger isomer, and will be stronger than if it were completely resolved. If z mol of the partly resolved form of the weaker produce the same biological effect

as 1 mol of the pure more active isomer, the observed stereospecificity, z, will be less than the true stereospecificity, x, and

$$\frac{1}{z} = \frac{y}{x} + (1 - y) \text{ so } x = \frac{y}{\frac{1}{z} - (1 - y)} \text{ and } z = \frac{1}{\frac{y}{x} + (1 - y)}$$

For example, if y = 0·5, i.e. for a racemic mixture, z will lie between 1 (when x = 1) and 2 (when x is very large).

For the piperidino-compounds, 247C53 and 248C53, the observed value for z is 5·5, so if y were 0·95, the correct value for the stereospecificity would be 7·2, for y = 0·90 it would be 11 and y = 0·85 it would be 26·6. The limiting value of y, corresponding to one isomer being completely inactive (x = ∞) is 0·818.

The optical rotations are shown in Table 2. The specific rotations for the pyrrolidino-compounds, 363C52 and 364C52, are very close to the values obtained by Duffin

Table 2. *Molar rotations obtained with a 5 cm cell.* For 247C53 in chloroform the concentration was 6·35 × 10^{-3}M; for 248C53 in chloroform it was 10^{-2}M; for all other measurements it was 2 × 10^{-2}M (and the figures indicate the actual angles measured in millidegrees, corrected for the solvent blank). Each figure is the mean of four sets of measurements, except for those marked with an asterisk, which are the means of only three sets. The standard error shown is based on the combined variance of the values for solution and for solvent and may be an underestimate (see text).

	Wavelength, nm				
	546	320	300	290	280
363C52 in $CHCl_3$ ..	+103	+421	+527	+607	+740
	±1·8	±5·1	±5·6	±6·3	±4·3
in water ..		−20	−34	−44	
		±1·5	±1·1	±0·9	
364C52 in $CHCl_3$ ..	−102	−411	−517	−598	−723
	±1·6	±2·6	±2·3	±2·5	±3·3
in water ..		+15	+29	+37	
		±1·2	±2·4	±0·7	
247C53 in $CHCl_3$ ..	+99	+480	+644	+768	+894
	±1·8	±5·2	±6·4	±10·7	±5·7
in water ..		−48*	−66*	−77*	
		±1·5	±2·1	±4·2	
248C53 in $CHCl_3$ ..	−126	−505	−642	−738	−908
	±1·7	±3·3	±4·4	±4·8	±4·4
in water ..		+54*	+74*	+84*	
		±0·9	±0·9	±2·2	
427C52 in $CHCl_3$ ..	−104	−490	−647		
	±1·4	±3·0	±2·4		
in water ..	−17	−63	−77	−104	
	±1·2	±0·8	±0·9	±1·7	
250C53 in $CHCl_3$ ..	−99	−458	−585		
	±1·9	±1·5	±2·8		

The specific rotations in chloroform at 546 nm, with the values obtained by Duffin & Green (1955) shown in parentheses, are:

363C52, +31·8 (+30); 364C52, −31·5 (−30); 247C53, +29·3 (+31); 248C53, −37·3 (−30); 427C52, −24·4 (−25); 250C53, −22·4 (−22).

& Green (1955). The values for the piperidino-compounds are less satisfactory; that for 248C53 is higher than the recorded value and that for 247C53 slightly lower. If the rotations obtained with the biggest measured angles (in chloroform at short wavelengths) are compared, the resolution would appear to have been effective. The rotations are much higher than those for the pyrrolidino-compounds, and these must have been resolved satisfactorily (see below). If the values for the rotations at 546 nm are used to assess the purity, however, the observed rotation, 99°, instead of a rotation of 126°, indicates that the (+)-isomer is a fraction 225/252 = 0·89 pure. If the resolution were only complete to this extent, the true stereospecific index would be 11·9, but this is still a very low figure compared with the value for the pyrrolidino-compounds, 375.

For a true stereospecificity (x) of 100, the observed value, 5·5, would be obtained only when the material was 82·6% pure. The (−)-isomer could, of course, also be impure. This would affect the stereospecificity slightly and if it, too, were only 82·6% pure the difference between the pure isomers would be 100/0·826 = 122; i.e. with both (+)- and (−)-isomers only 82·6% pure, the observed stereospecific index of 5·5 would be obtained when the difference between the pure isomers was 122. In this situation, however, the size of the rotations would be equal for the two isomers; this illustrates the difficulty of using optical rotations to assess purity.

The very high stereospecificity of some of the compounds, however, does indicate their purity. For the pyrrolidino-compounds, 363C52 and 364C52, the value for z is 375 and the limiting value of y, corresponding to one isomer being completely inactive (x = ∞), is (1–0·0026), i.e. this material must be at least 99·74% pure. The biological effects of the compounds are produced by antagonizing the actions of acetylcholine and there is little likelihood that the isomers are interacting with each other either chemically or biologically.

If it is argued that the low stereospecificity of the piperidino-compounds 247C53 and 248C53 is due to inadequate resolution, it is possible to obtain an idea of the size of the rotations of the pure compounds. If 247C53, the material with the smallest rotation (M_{546} + 99), were only 82·6% pure, the value for the pure material would be +152 (α_{546} + 44·9). This is much in excess of the values for the pyrrolidino-compounds, 363C52 and 364C52, which must be pure because they have high stereospecificity. With the metho-compounds, 427C53 and 250C53, which also have high stereospecificity, the change from pyrrolidino to piperidino leads to a slight decrease in rotation. It seems, therefore, very unlikely that the rotations of the piperidino compounds 247C53 and 248C53 could be as high as they would need to be if their low stereospecificity were due simply to inadequate resolution.

The results show that the more active isomer is (−)-rotatory in chloroform. The more active isomers of the esters of phenylcyclohexylglycollic acid studied by Ellenbroek & others (1965) were also (−)-rotatory but were tested in solution in methanol; they were all quaternary salts. The absolute configuration of these follows from the synthesis of the phenylcyclohexylglycollic acids and their methyl esters from starting materials of known absolute configuration by Inch & others (1968), who measured the rotations of these compounds both in methanol and in chloroform. As the rotations of the phenylcyclohexylhydroxypropyl compounds tested here did not change sign at shorter wavelengths it seems likely that the more active (−)-isomers have the same absolute configuration (*R*) as the more active (−)-isomers of the esters, even though they contain methylene groups and not a carboxyl group attached

to the asymmetric centre. The configuration cannot be regarded as being certain, however, particularly in view of the effects of solvent on the rotations of this type of compound, which with water have been found even to lead to a change in the sign of rotation.

DISCUSSION

These results confirm the findings of Duffin & Green (1955) that there can be large differences in the stereospecificity of closely related compounds. Any attempt to make deductions about receptor structure must therefore be limited to situations in which many pairs of compounds have been tested. A false impression of the asymmetry of the acetylcholine receptor would be obtained from considering only the piperidino-compounds, for instance.

These particular results also illustrate how variable the effects of a group are on affinity and the need to consider how far the contribution of the group to binding may be offset by rearrangement of the binding of the rest of the molecule. The stereospecificity of the pyrrolidino-compounds, for instance, indicates a difference in the free energy of adsorption ($-\Delta G = RT\ln K$) of 3·65 kcal mol^{-1} (15·3 kJ mol^{-1}), which might be ascribed to hydrogen bonding of the hydroxyl group to the receptor in the (−)-isomer and not in the (+)-isomer, but smaller differences are obtained with the other pairs of compounds. In fact, it is highly unlikely that the difference between the isomers is only due to extra binding by the hydroxyl group in the (−)-compounds (see below). The change from pyrrolidine to piperidine in the (+)-isomers increases log K from 5·69 to 8·01, indicating an increase in the free energy of adsorption of 3·29 kcal mol^{-1} (13·8 kJ mol^{-1}) and it is difficult to see how such a large increase can come simply from binding of the extra methylene group in the piperidine ring. The unexpectedly large effect of the change can be compared with the unexpectedly large effects of introducing the cyclohexyl group into phenylacetyl and phenylglycolloyl esters (Abramson & others, 1969). It could be interpreted by supposing that the binding of the rest of the molecule in the (+)-series of compounds is disturbed much less by the introduction of a piperidino-group than by the introduction of a pyrrolidino-group. Unfortunately, there are not enough results to make it possible to estimate the effects of the change in structure from pyrrolidino- to piperidino-group on the binding of the rest of the molecule (as was possible for the introduction of phenyl and cyclohexyl groups in the compounds referred to above). It seems likely however, that they are large and also that the difference between affinity of optical isomers will be the result of differences in the contributions to binding of all the groups in the molecule, not just of one group (such as the hydroxyl group) in these compounds.

The irregular effects of quite simple changes in structure on affinity can also be seen by considering the replacement of hydrogen by methyl and methyl by ethyl in these compounds. In three instances the replacement of hydrogen by methyl increased affinity (between 2 and 12-fold) but it decreased the affinity of the (+)-isomer of the piperidino-compounds nearly 6-fold. Replacement of methyl by ethyl reduced the affinity of the pyrrolidino-compounds slightly [just over 2-fold in the (+)-isomers and not significantly in the (−)-isomers] but increased the affinity of the (−)-isomer of the piperidino-compounds to the extremely high value of 9·73. The size of these changes is again so large that it is unlikely that they can be ascribed simply to binding of the extra methylene group to the receptors, or its failure to bind. It is necessary

to consider also the effect of the extra methylene group on the binding of the rest of the molecule.

There is no evidence that stereospecificity is greater with the more active compounds as has been suggested by Pfeiffer (1956) or Ariëns, Simonis & van Rossum (1964). With the compounds used in the present work the reverse is true; those with lower affinity have higher stereospecificity. It seems probable that it is not possible to generalize at all because each type of drug and receptor will constitute a separate problem.

Acknowledgements

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