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The relation between biological activity and the degree of resolution of optical isomers

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Previous reports of the low stereospecificity of benzhexol can be ascribed to inadequate resolution of the samples tested and a report of much higher stereospecificity has been confirmed. The two enantiomers have been found to differ over 1000-fold in their affinity for the postganglionic acetylcholine receptors of the guinea-pig ileum. Mixtures of the enantiomeric forms of phenylcyclohexylglycolloylcholine and of benzhexol have been tested on this preparation and the dose-ratios used to calculate apparent affinity constants. With both pairs the results indicate that the two enantiomers compete with the agonist and with each other and justify the use of the stereospecific index to set limits to the degree of resolution. For compounds such as these, in which one enantiomer has appreciably higher biological activity than the other, this biological method for assessing stereochemical purity is likely to be at least as satisfactory as any nuclear magnetic resonance method currently in use, because of the very great sensitivity of the stereospecific index to the degree of resolution.

In a paper on the stereospecificity of closely related pairs of enantiomers it was pointed out that the ratio of the activities of enantiomers (the stereospecific index) depends greatly on the degree of resolution that has been achieved (Barlow, 1971). It is not possible to assess this with confidence from the optical rotations because it is impossible to know with certainty what the values should be for completely resolved material. With compounds that are active biologically, and where the activity is stereospecific, it was claimed that the stereospecific index may be used to set a lower limit to the degree of resolution. For example, the enantiomeric forms of procyclidine are both antagonists of acetylcholine and the stereospecific index was calculated to be 375 from measurements of their affinity constants for the postganglionic acetylcholine receptors of the guinea-pig ileum. If one isomer is completely inactive, it was suggested that a value as high as this could only be obtained if the sample of this form were 99·7% optically pure and as it is unlikely to be completely inactive the optical purity of the sample should actually be higher than this.

The stereospecificity of benzhexol was of particular interest because it is low. For the postganglionic acetylcholine receptors of the guinea-pig ileum the results of Duffin & Green (1955) indicated a stereospecific index of 9·8 and from the affinity constant measurements with samples of the same material the value was 5·5 (Barlow, 1971). Long, Luduena & others (1956) also tested the resolved forms of pipanol (benzhexol) and obtained results that indicated a much greater stereospecificity. For instance, in tests as antagonists of acetylcholine on rabbit ileum the ratio of the the activities of the two enantiomers was 160:1 and as they were only allowed to act for 2 min this is likely to be an underestimate. Activity in this situation would

depend greatly on rates of diffusion, which should be the same for the two isomers.

Thanks to the kindness of Dr. F. P. Luduena and Dr. F. C. Nachod we have been able to test samples of the material used by Long & others (1956). We found that the affinity constants for postganglionic acetylcholine receptors of the guinea-pig ileum indicated a stereospecific index of 1190, so clearly the resolution of the isomers of benzhexol as described by Adamson & Duffin (1957) was incomplete. Comparison of the optical rotations of the samples indicated that both forms of benzhexol tested by Duffin & Green were 87–90% optically pure but their sample of the weaker (+)-form had an affinity constant 240 times that of the sample studied by Long & others (1956) and if the latter sample is optically pure, the former would appear to be only about 80% resolved.

The discrepancy between the two estimates of optical purity, 80% from the biological results and 90% from the rotations, is large enough to imply that there may be a serious limit to the confidence which can be placed in estimates of the degree of resolution calculated from values of the stereospecific index. It will partly be due to the experimental error of the biological results but it also seemed possible that it might have been incorrect to assume that the biological activity of a mixture of enantiomers is the sum of the activities of its components. It was possible, for instance, that there was interaction between the biological effects of the two forms (although this seemed unlikely because both appeared to be competing with acetylcholine for the receptors). We thought it important, therefore, to investigate experimentally the effect of enantiomeric composition on the biological activity of compounds of this type. To do this we have made up a range of mixtures of the *R*- and *S*-forms of phenylcyclohexylglycolloylcholine and of the (+)- and (−)-forms of benzhexol and measured the affinity of these mixtures for the acetylcholine receptors of the guinea-pig ileum.

If two compounds are acting as competitive antagonists of acetylcholine, the dose-ratio observed with the two acting together is $DR_1 + DR_2 - 1$, where DR_1 and DR_2 are the dose-ratios produced by each concentration of antagonist acting separately (Paton & Rang, 1965; Abramson, Barlow & others, 1969). If the compounds are present in concentrations B and C and have affinity constants K_B and K_C, respectively,

$$DR_1 + DR_2 - 1 = 1 + BK_B + CK_C.$$

If the compounds act non-competitively the combined dose-ratio is $DR_1 \times DR_2$.

For a mixture containing a concentration B of antagonist of which a proportion y_s is the stronger isomer, the dose-ratio observed $= 1 + (1 - y_s)BK_w + y_sBK_s$, where K_w and K_s are the affinity constants of the weaker and stronger respecting forms; the apparent affinity constant, $K^* = (1 - y_s)K_w + y_sK_s$. Accordingly, even though the two forms of the compound compete with each other as well as with the agonist, the apparent affinity constant should be the sum of the contributions from the two forms. The graph of K^* against y_s should be a straight line because

$$K^* - K_w = y_s (K_s - K_w)$$

and as large changes in apparent affinity occur when the proportion of more active isomer is small, a more sensitive test is to plot log $(K^* - K_w)$ against log y_s, which should give a straight line with a slope of unity.

MATERIALS AND METHODS

Compounds. The resolved forms of benzhexol were samples of the material tested by Long & others (1956). They resolved the base with *N*-benzoyl-D-threonine

(Adamson & Duffin, 1957, used (+)-tartaric acid) and continued the resolution until the biological activity of the weaker isomer did not decrease any further.

The resolved forms of phenylcyclohexylglycolloylcholine iodide were obtained by esterifying resolved forms of phenylcyclohexylglycollic acid. The ester from the *R*(−)-acid had m.p. 141·6–142·4°, M_D (5×10^{-2}M in methanol) + 23·6; α_D + 5·28°; I^-, 28·20; that from the *S*(+)-acid had m.p. 140·8–142·0°; M_D −23·2°; α_D−5·19; I^-, 28·05; I^- (theory), 28·35. Further details of these compounds will appear elsewhere. The signs of the rotations of these compounds dissolved in methanol (Analar: <0·1% water) change at shorter wavelengths and become the same as those of the parent acids and this leads to some confusion. The ester from the *R*-(−)-acid is (+)- at the *D*-line in this solvent and we have therefore referred to the enantiomers only by the sign of their absolute configuration. Ellenbroek, Nivard & others (1965) recorded α_D in methanol of −5·3 and +6·1 for the *R* and *S* isomers, respectively. We think it likely that these have inadvertently been allotted the sign of rotation of the parent acids. The signs of the rotations of the compounds in solution in water (and in chloroform) are the same as those of the parent acids so an alternative explanation is that Ellenbroek & others used wet methanol.

Optical rotations were measured with a Bellingham and Stanley Model B spectropolarimeter (Barlow, 1971). Values quoted are the mean and standard error of four estimates made with the same solution. This is an underestimate of the real error which includes appreciable errors in the preparation of the solutions, as well as errors due to small differences in the temperature at which the measurements were made. From repeated measurements with different solutions of the same material the error attached to the mean value appears to be between 1 and 2%.

Affinity constant measurements. These were measured on the isolated guinea-pig ileum at 37° with carbachol as the agonist (Barlow, 1971; Abramson & others, 1969). The tissue was suspended in Tyrode solution containing hexamethonium, $2{\cdot}76 \times 10^{-4}$M, through which air was blown, and the contractions of the muscle were recorded isotonically.

RESULTS

The optical rotations of the samples of the enantiomeric forms of benzhexol are shown in Table 1, together with the means of the estimates of logarithm of the affinity constant for the postganglionic acetylcholine receptors of the guinea-pig ileum. The optical purity of the samples has been calculated assuming that the size of the rotations of the (−)-form studied by Long & others (1956) is that of completely resolved material. The values include two estimates of the rotations of the (+)-form which were obtained with different solutions of the same material. These give some indication of the real errors, 1 to 2%, attached to these figures, which exceed the statistical errors involved in operating the instrument. The situation is the reverse of that with an optical instrument in which the errors of observation are greater than those involved in making up the much stronger solutions which are needed.

The optical purity of the more active (−)-isomer will have only a small effect on its biological activity and the less pure sample (marked D and G in Table 1) appears to be slightly more active than the optically purer material but the difference between the two estimates of log K is only 0·05 log units and is within the expected limits of error. In this type of experiment, the repetition of measurements with fresh stock solutions by different workers at different times has shown that there

are systematic errors which are slightly greater than would be predicted by the variance of estimates within any one set of observations. Abramson & others (1969) concluded that 'differences of the order of 0·1 log units were likely to indicate real differences' between the means of a group of estimates of log K and in so far as it is possible to check from the results in the present work, this seems to be a reasonable conclusion. The two estimates for the same sample of the (+)-form of benzhexol, for instance, shown in Table 1 differ by 0·075 log units and the value of log K for *R*-phenylcyclohexylglycolloylcholine shown in Table 2 differs by only 0·013 log units from that published by Brimblecombe, Green & others (1971). Mean estimates of affinity constants based on the numbers of experiments made in this work (with 5–10 pieces of ileum) can therefore be regarded as likely to lie within the range 0·8 to 1·26 times the antilog of the mean log K.

The results of the experiments with mixtures of the *R*- and *S*-forms of phenylcyclohexylglycolloylcholine are shown in Table 2A. In these experiments each mixture was tested in concentrations which produced dose-ratios of between 20 and 90, and all except the 0·0 and 0·1% were also tested in higher concentrations, which produced dose-ratios of between 100 and 900. The mean values of log K are therefore based on values from a wide range of dose-ratios. The graph of the apparent affinity constant (K*) against the proportion of the *R*-form (y_S) is a straight line (Fig. 1). The graph of log ($K^* - K_W$) against log y_S is also linear (Fig. 2A) and it can be seen that this arrangement makes it possible to observe the fit of the

Table 1. *Optical rotations and biological activity of samples of benzhexol.* Values for the molar rotation are the means of four estimates (with the same solution) and the standard error is shown.*

			Molar rotation ± s.e. (4 estimates)					Log K (ileum)
		α546	546 nm	320	300	290	280	
(−) L, L, T and L		45·6	154	646	823	946	1138	8·700 ± 0·024 (7)
in $CHCl_3$			±1·0	±1·7	±2·4	±2·7	±2·2	
D and G		37·3	126	505	642	738	908	8·751 ± 0·015 (7)
Optical purity %			90·9	89·1	89·0	89·0	89·9	
L, L, T and L			+13	72	97	110		
in water			±1·0	±1·2	±0·8	±1·8		
D and G				54	74	84		
(+) L, L, T and L	(i)	42·9	145	622	798	929	1104	5·625 ± 0·032 (7)
in $CHCl_3$			±0·9	±0·7	±1·2	±3·8	±1·6	
	(ii)		146	634	810	936	1122	5·700 ± 0·038 (5)
Optical purity %	(i)		97·1	98·1	98·5	99·1	98·5	
D and G		29·3(?)	99	480	644	768	894	8·008 ± 0·033 (9)
in $CHCl_3$								
Optical purity %			82·1	87·1	89·1	90·6	89·3	
L, L, T and L			−13	68	92	108		
in water			±1·0	±1·9	±0·8	±0·8		
D and G				48	66	77		

* This is an underestimate of the real error which is 1 to 2% and can be assessed by comparing the two values for the (+)-form marked (i) and (ii), which were obtained with different solutions made from the same material. The samples studied by Duffin & Green (1955) are marked 'D and G' and the results are those obtained by Barlow (1971). The samples studied by Long & others (1956) are marked 'L, L, T and L'. They recorded α_D −38·4° and +38·3° in chloroform. The optical purity has been calculated on the assumption that the sample of (−)-benzhexol marked L, L, T and L has been completely resolved. Note that the signs of the rotations in water are the opposite of those in chloroform.

The logarithms of the affinity constants for the postganglionic acetylcholine receptors of the guinea-pig ileum are shown with the standard error and the number of estimates.

The stereospecific index for the material marked L, L, T and L is $10^{8·70-5·62} = 1190$.

results to the relation over a wider range of values. Clearly these particular enantiomers behave in a manner consistent with the theory and the results justify the use of the theory to infer limits of optical purity from values of the stereospecific index.

The results with the enantiomeric forms of benzhexol are shown in Table 2B. In these experiments each mixture was tested in a concentration that produced dose-ratios lying in a narrow range (between 21 and 31). The graph of log ($K^* - K_w$) against log y_s shows that the biological activity of these mixtures, like those previously studied, is in agreement with the theory based on their action as competitive antagonists. If either compound were acting non-competitively the dose-ratios of the mixtures would be higher (Table 2B) and the expected values of K* for the particular concentrations used in these experiments are shown in Fig. 2B. It is difficult to distinguish between competitive and non-competitive behaviour with mixtures containing higher proportions of the more active isomer, unless these were tested in high concentrations so that the amount of less active isomer produces an appreciable dose-ratio. With the lower proportions of the more active isomer, however, the differences between the results expected from non-competitive behaviour and from competitive behaviour are well outside the limits of experimental error.

DISCUSSION

Stereospecific index and optical purity

With two pairs of enantiomers we have evidence that the biological activity, assessed by the affinity constant for the receptors, is the sum of the contributions from the two forms present. For a sample of the stronger isomer containing a proportion ($1 - y_s$) of the weaker isomer, the observed affinity constant

$$K_s^* = y_s K_s + (1 - y_s)K_w,$$

Table 2. *Effect of composition on affinity constants (guinea-pig ileum) of enantiomeric mixtures.* The percentage of the stronger isomer present in the mixture (y_s %) is shown with the mean estimate of log affinity constant (log K*) ± the standard error and number of results.

A		B		Dose-ratios			
y_s %	Log K*	y_s %	Log K*	DR_w	DR_s	comp.	non-comp.
0	7·130 ± 0·037 (6)	0	5·700 ± 0·038 (5)	—	—	—	—
0·1	7·252 ± 0·023 (4)	0·1	5·563 ± 0·019 (3)	11	11	21	121
—	—	0·2	6·126 ± 0·033 (2)	—	—	—	—
0·5	7·570 ± 0·036 (5)	0·5	6·380 ± 0·036 (4)	6	26	31	156
1·0	7·737 ± 0·055 (7)	0·99	6·680 ± 0·025 (4)	3·5	26	28·5	91
5·0	8·360 ± 0·033 (6)	4·8	7·190 ± 0·061 (6)	1·5	26	26·5	39
10·0	8·642 ± 0·054 (7)	9·1	7·632 ± 0·046 (4)	1·25	26	26·25	32·5
20·0	8·886 ± 0·029 (11)	16·7	7·932 ± 0·033 (4)	1·1	21	21·1	23·1
50·0	9·369 ± 0·026 (5)	50·0	8·354 ± 0·031 (4)	1·03	31	31·03	31·1
100	9·647 ± 0·073 (11)	100	8·700 ± 0·024 (7)	—	—	—	—

Section A refers to experiments with phenylcyclohexylglycolloylcholine iodide and Section B to experiments with benzhexol. DR_w and DR_s indicate the dose-ratios which the concentrations of the weaker and stronger enantiomers of benzhexol should produce alone, the column marked 'comp.' their combined dose-ratio if they act competitively and that marked 'noncomp.' the combined dose-ratio if either acts noncompetitively.

Brimblecombe & others (1971) obtained estimates of 7·38 ± 0·02 (6) and 9·66 ± 0·08 (6) for their samples of *S*- and *R*-phenylcyclohexylglycolloylcholine, respectively, and Abramson & others (1969) obtained an estimate of 9·365 ± 0·033 (7) for the racemate. The value for the (+)-form of benzhexol should be compared with an earlier estimate, obtained with the same sample, 5·625 ± 0·032 (7) (see Table 1).

where K_S and K_W are the affinity constants for the pure forms of the stronger and weaker enantiomer respectively.

For a sample of the weaker isomer, containing a proportion $(1 - y_W)$ of the stronger isomer, the observed affinity constant

$$K_W^* = y_W K_W + (1 - y_W)K_S$$

and the observed stereospecific index, $S^* = \frac{K_S^*}{K_W^*} = \frac{y_S K_S + (1 - y_S)K_W}{y_W K_W + (1 - y_W)K_S}$

If $K_S \gg K_W$ and $y \to 1$, the apparent affinity constant of the stronger isomer $= y_S K_S$, because the contribution from the small amounts of weaker isomer present is negligible, and the expression can be rewritten

$$\frac{1}{S^*} = \frac{y_W}{y_S}\frac{1}{S} + \frac{(1 - y_W)}{y_S}$$

where S is the true stereospecificity, $\frac{K_S}{K_W}$.

If the sample of stronger material is regarded as pure ($y_S = 1$) the expression reduces to the form previously described (Barlow, 1971) but it is difficult to be sure that the material is as pure as this. The situation usually met is that the two forms have rotations of roughly equal size and opposite sign, i.e. $y_S = y_W$ but does not necessarily equal unity. This applies, for example, to the two forms of benzhexol studied by Duffin & Green. The expression then becomes $\frac{1}{S^*} = \frac{1}{S} + \frac{1 - y}{y}$ where $y = y_S = y_W$. The relation between the degree of resolution, y, and the observed

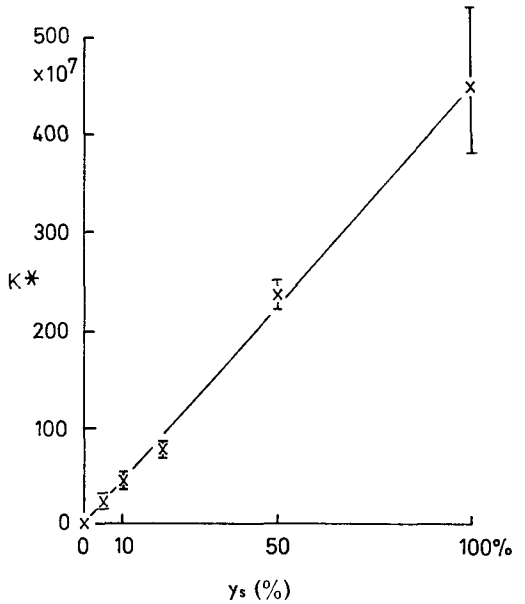

FIG. 1. Results with mixtures of the enantiomeric forms of phenylcyclohexylglycolloylcholine. The apparent affinity constant, K*, is plotted against the proportion of the stronger isomer present in the mixture (y_S). Although the exact proportion is not known (because the 'pure' forms may not be completely resolved), it cannot differ greatly from the value indicated because an appreciable degree of resolution has clearly been achieved. The bars indicate the standard errors of the estimates of the affinity constant and these have been calculated on the assumption that it is estimates of log K which are normally distributed.

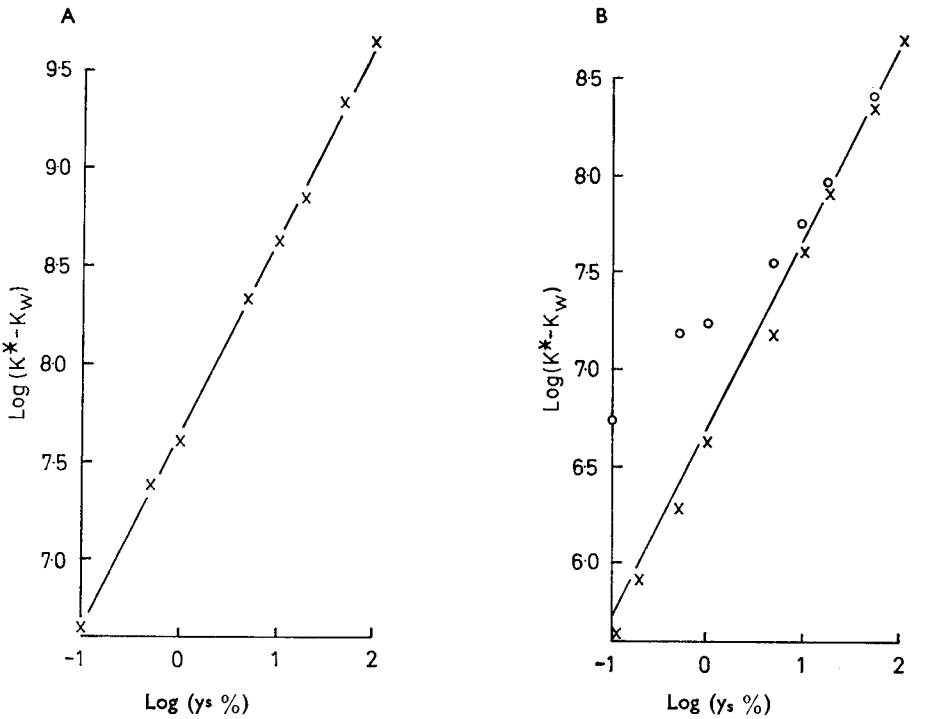

FIG. 2A. The logarithm of (the apparent affinity constant, K*, − the affinity constant of the weaker isomer, K_w) is plotted against the logarithm of the proportion of the stronger isomer present in the mixture (y_s). The best line has been drawn by eye to fit the points.

B. Results with mixtures of the enantiomers of benzhexol. Log (K^*-K_w) is plotted against log y_s, as in Fig. 2A. The line corresponds to the expected values when log K_w = 5·70 and log K_s = 8·70 (Table 2A). A better fit is obtained with the earlier estimate of log K_w (5·62, Table 1). This alters the values of log (K^*-K_w) as well as the position of the line. The open circles indicate the apparent affinity constants which would be expected if one enantiomer acts non-competitively. These will depend on the concentrations tested and the values shown are for the concentrations listed in Table 2A.

stereospecific index, S*, for particular values of the true stereospecific index, S, is shown in Fig. 3.

This shows the very striking dependance of stereospecificity on optical purity. If the isomers are only 95% resolved, the highest stereospecific index which could theoretically be observed, corresponding to a true stereospecific index of infinity (with one enantiomer completely inactive), would be 19. On the other hand, if a value of 100 is obtained for the stereospecific index experimentally then the degree of resolution should be better than 100/101, i.e. 99·01% and a value of 1000, such as we have obtained with the forms of benzhexol studied by Long & others (1956), indicates that these should be at least 99·9% resolved.

It is necessary to see how far these estimates of the degree of resolution should be revised to allow for errors in the estimation of the stereospecific index. If the errors in an estimate of log K in our experiments are 0·1 of a log unit, the errors in the logarithm of the stereospecific index should be of the order of 0·2 log units because log S = log K_s − log K_w. An experimental value for the stereospecific index of 100 might therefore be considered to indicate a range from 63 to 158, and the minimum degree of resolution might therefore be as low as 63/64 = 98·5%.

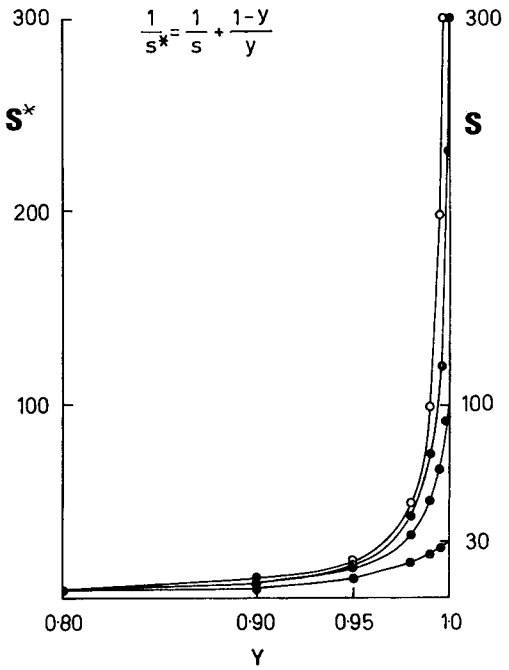

FIG. 3. The effect of the degree of resolution on the stereospecific index. The stereospecific index observed, S*, is plotted against the degree of resolution, y, for true values of the stereospecific index (S) of 30, 100, 300 and infinity (open circles). Note the great sensitivity of S* to incompleteness of resolution. These values have been calculated on the assumption that both enantiomers are resolved to the same extent (y).

The results obtained with the samples of benzhexol studied by Duffin & Green may be regarded as an extreme instance of the variation likely to be observed with estimates of stereospecific index. From Duffin & Green's estimate of the stereospecificity, 9·8, the degree of resolution should be 91%; the values of the rotations (Table 1) suggest that this is an overestimate and the value is nearer 89%. The stereospecific index recorded by Barlow (1971) was 5·5 and if allowance is made for possible errors this could be as high as 8·7, which corresponds to a degree of resolution of 89·7%. The fit of the results to the line in Fig. 2A and B provide further evidence that in this particular test the uncertainty in the stereospecific index is likely to be by a factor of about 1·6. The divergence of the results in Fig. 2B from linearity, for example, appears to arise from an error of 0·075 in the estimate of log K for the weaker form of benzhexol and if the errors in the estimates of mean values of log K were much greater than this the results would be strikingly different from the expected values.

Several methods have recently been developed for assessing the degree of resolution of enantiomers because it is possible in certain circumstances to distinguish between nuclear magnetic resonance signals from the two forms (Pirkle, 1966; Raban & Mislow, 1966; Dale, Dull & Mosher, 1969; Goering, Eikenberry & Koermer, 1971). They might therefore be of great value for predicting the true stereospecificity of enantiomers from samples that are incompletely resolved, but it is clear from Fig. 3 that they can only do this if they capable of distinguishing between 99·0 and 99·5% resolution. Although in some instances an accuracy of $\pm 1\%$ has been claimed, it remains to be seen whether any method is really accurate enough for it to give

reliable estimates of true stereospecificity from results with incompletely resolved samples. It would seem necessary to perform experiments, similar to those described here, in which the biological activity of mixtures of enantiomers is compared with their stereochemical purity assessed by an nmr method. For the rather limited class of compounds like benzhexol, which have a high stereospecificity and whose biological activity can be measured particularly accurately, it seems that biological methods may still continue to be useful for assessing the limits of the degree of resolution, although they may eventually be replaced by physical techniques.

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