the view that β-adrenoceptor blockade alone can reverse the metabolic reactions to severe stress.

This work was supported by grants from the Joint Research Board of St Bartholomew's Hospital and the Servier Laboratories. Grateful thanks are due to Dr P. V. Cole of the Department of Anaesthetics who provided technical services.

March 7, 1977

REFERENCES

BALL, J. H., KAMINSKY, N. I., HARDTMAN, J. G., BROADUS, A. E., SUTHERLAND, E. W. & LIDDLE, G. W. (1972). *J. clin. Invest.*, **51**, 2124–2129.

BROWN, B. L., ALBANO, J. D. M., EKINS, R. P., SCHERZI, A. M. & TAMPION, W. (1971). *Biochem. J.*, **121**, 561–562.

BUTCHER, R. W. & BAIRD, C. E. (1969). In: *Drugs affecting lipid metabolism.* Editor: Paoletti, R. New York: Plenum Press.

CARRUTHERS, M. E. (1969). *Lancet*, **2**, 1170–1171.

CARRUTHERS, M. E. & YOUNG, D. A. B. (1973). *Clinica chim. Acta*, **49**, 341–348.

ELIASCH, H., ROSEN A. & SCOTT, H. M. (1967). *Br. Heart J.*, **29**, 671–683.

EXTON, J. H. & PARK, C. R. (1968). *Adv. Enzyme Regul.*, **6**, 391–407.

FRANKENHAUSER, M. (1971). In: *Society, Stress and Disease*, Editor: Levi, L. Oxford: University Press.

GILBERT, C. H. & GALTON, D. J. (1974). *Horm. Metab. Res.*, **6**, 229–233.

IMHOF, P. R., BLATTER, K., FUCELLA, L. M. & TURRI, M. (1969). *J. appl. Physiol.*, **27**, 266–371.

MCCULLOUGH, H. (1968). *J. clin. Path.*, **21**, 759–763.

SUTHERLAND, E. W. & ROBISON, G. A. (1966). *Pharmac. Rev.*, **18**, 145–161.

WHITE, J. E. & ENGEL, F. L. (1958). *J. clin. Invest.*, **37**, 1556–1563.

Benzodiazepine activity: is interaction with the glycine receptor, as evidenced by displacement of strychnine binding, a useful criterion?

PETER HUNT*, JEAN-PIERRE RAYNAUD, *Centre de Recherches Roussel Uclaf, 93230 Romainville, France*

Young & Snyder (1973) have clearly demonstrated that specific [^{3}H]strychnine binding to spinal cord synaptic membranes *in vitro* is associated with the glycine receptor. A correlation has also been made (Young, Zukin & Snyder, 1974) between the capacity of benzodiazepines to displace [^{3}H]strychnine binding and their potency in pharmacological and behavioural tests considered as the most relevant to clinical efficacy. The authors thus suggest that benzodiazepines may exert their anxiolytic, myorelaxant and anticonvulsant effects by mimicking the effects of glycine at its central receptor sites.

The present report is concerned with the usefulness and the specificity of the *in vitro* system for the evaluation of benzodiazepines and as a criterion for benzodiazepine-like activity.

Specifically labelled [15-^{3}H]strychnine, specific activity 11·2 Ci mmol^{-1} was prepared at Roussel-Uclaf. The labelled material behaved identically with the authentic compound on thin-layer chromatography in two different systems: on alumina (Merck, T) with benzene–ethanol (9·8 : 0·2) and on silicagel (Merck, GF 254) with chloroform–methanol–aqueous ammonia (9 : 1 : 0·2).

The preparation and incubation of synaptic membranes from rat (Sprague Dawley strain, male, 150–200 g) spinal cord and pons-medulla was carried out following essentially the method of Young & Snyder (1973). The membrane pellets, which were conserved at —28°, were resuspended in 0·05 M Na, K-phosphate buffer, pH 7·2, containing 200 mM NaCl, to give a protein concentration of 0·25–0·5 mg ml^{-1}. The suspension, in aliquots of 2 ml, was incubated in the presence of [^{3}H]strychnine (10^{-8}M) and different compounds at various concentrations for 20 min at 4°. Benzodiazepines were dissolved in ethanol so that the final concentration of ethanol in the incubation mixture was 1%. Ethanol concentrations up to at least 2·5% had no effect on specific [^{3}H]strychnine binding. The incubate was filtered on Whatman glass fibre discs, type GF/C, using a Millipore apparatus and the filters were washed rapidly (washing time less than 10 s) with 10 ml of an ice-cold solution of 0·15 M NaCl. The radioactivity on the filters was measured after digestion overnight in scintillation fluid containing 30% Triton X-100. Using the filtration technique, non-specific (NS) binding of [^{3}H]strychnine to membranes, which is defined as that not displaced by a high concentration of strychnine (10^{-4}M) or glycine (10^{-2}M), is completely eliminated. Residual binding, which amounts to about 6% of the total on average, is independent of membrane protein concentration and is accounted for by [^{3}H]strychnine binding to the glass-fibre filters. NS binding in the centrifugation technique of Young & Snyder (1973) represents 20–30% of the total. In both techniques, however, specific binding (that which is displaced by 10^{-2}M glycine) is identical and is equal to, on average,

* Correspondence.

0·37 pmol of strychnine bound mg^{-1} of protein. [^{3}H]-strychnine is displaced by unlabelled strychnine or glycine and the concentrations (IC50) causing half-maximal displacement (strychnine = $1·0 \times 10^{-8}$ M; glycine = $1·8 \times 10^{-5}$ M) agree well with those reported (Young & Snyder, 1973; Young & others, 1974). For a number of 1,4-benzodiazepines, however, IC50 values are higher (3–6 times) than those reported (Table 1a). The centrifugation technique, in our hands, gives somewhat lower values (Table 1a) but the order of activity is similar. The difference may be explained by the presence or absence of NS binding. We found that with the centrifugation technique NS binding of [^{3}H]strychnine in the presence of 10^{-4}M strychnine (~12% of the total) was less than that in the presence of 10^{-2} M glycine (~ 20% of the total). In other words, strychnine maximally displaces more [^{3}H]strychnine than does glycine, probably indicating a minor component of strychnine binding which is saturable at high concentrations and which is not associated with glycine binding. When benzodiazepines were incubated, simultaneously with 10^{-2} M glycine, in concentrations of the order of their IC50 (eg. $2·5–5 \times 10^{-5}$ M for diazepam and nitrazepam) they reduced the level of 'glycine-NS' binding to close to that of 'strychnine-NS' binding. This effect is sufficient to account for the differences observed with the filtration technique where only the more specific binding is retained and where the benzodiazepines are evidently observed to be less active. The filtration technique should therefore give a better indication of the interaction of compounds with the more specific component of [^{3}H]strychnine binding, associated with the glycine receptor.

Table 1. *Displacement of specific [^{3}H]strychnine binding by benzodiazepines: comparison with liposolubility.*

	Displacement of [^{3}H]strychnine binding IC50 (μM)$^{(a)}$	Liposolubility $R_m(H_2O)^{(b)}$
Diazepam	87 (50)	2·0
Flunitrazepam	100 (50)	1·8
Nitrazepam	110 (55)	1·6
Alprazolam	150 (110)	1·5
Oxazepam	330 (250)	1·4
Chlordiazepoxide	630 (400)	1·4

(a) [^{3}H]Strychnine (10^{-9}M) is incubated with the homogenate (0·5 mg protein in 2 ml) in the presence of benzodiazepines at 3 different concentrations. IC50 values are determined by logarithmic probability plots of inhibitor concentration against percentage inhibition of displaceable [^{3}H]strychnine binding. Values represent the average of triplicate determinations in 2 separate experiments and varying by less than 10%. In brackets: values obtained using the centrifugation technique. Incubations contained 1 mg of protein in 2 ml.
(b) R_m (= $\log(1/R_F-1)$) is measured by thin-layer chromatography on silanized silicagel plates (Merck, 60 HF254) run in a mixture of acetone–water in varying proportions. R_m (average of 5 determinations) is plotted against the percentage of acetone in the mixture and extrapolated to 0% acetone to give $R_m(H_2O)$. The s.e.m. for the values is of the order of 0·1 R_m units.

While the number of compounds tested is insufficient for making correlations with biological or clinical activity, it is evident that *in vitro* activities cannot reflect some large differences in pharmacological activity, for example, between diazepam and flunitrazepam (Randall & Kappell, 1973) or alprazolam (Rudzik, Hester & others, 1973), the latter two being considerably more potent†. The IC50 values for the benzodiazepines are high and fall in the range where numerous lipophilic compounds exert non-specific effects on membranes. In order to test to what extent lipophilic interactions may be involved, an estimate of the liposolubility of the compounds was made by the measurement of their R_m values. As shown in Table 1b, liposolubility, as evaluated by this technique, parallels *in vitro* activity.

A further criterion for the specificity of the interaction of the benzodiazepines with the glycine receptor would be the lack of effect of compounds differing in chemical structure and pharmacological activity. In accordance with the results of Young & others (1974) a number of anticonvulsant or myorelaxant drugs were found to be devoid of activity at a concentration of 10^{-3} M (Table 2). One exception is thiocolchicoside, a centrally active muscle relaxant, active pharmacologically at very low doses (0·02 μg, sub-occipitally in frogs) (Plotka & Jequier, 1957). Its high activity in displacing [^{3}H]-

Table 2. *Displacement of specific [^{3}H]strychnine binding by tricyclic compounds and various anticonvulsant or myorelaxant drugs.*

		Displacement of [^{3}H]strychnine binding IC50 (μM)
	Thiocolchicoside	2·1
Tricyclic compounds:	Dibenzepine	14
	Chlorimipramine	93
	Amitriptyline	170
	Doxepine	180
	Chlorpromazine	190
	Protriptyline	190
	Imipramine	280
Compounds having no effect at 10^{-3}M:	Phenobarbitone	
	Diphenylhydantoin	
	Meprobamate	
	Mephenesin	
	Ethosuximide	

Incubation conditions and estimation of IC50 values were as described for Table 1a.

† Some pharmacologically inactive benzodiazepines, moreover, have *in vitro* activities which fall in the same range as the more active compounds tested (unpublished observations).

strychnine binding may correspond to either its myorelaxant properties or its convulsant properties which are manifested at higher doses.

It is most striking that various tricyclic derivatives are also effective in displacing [^{3}H]strychnine binding, having IC50 values of the same order or lower than those of the benzodiazepines (Table 2). As in the case of thiocolchicoside, this activity may reflect either convulsant or anticonvulsant properties. However, although the compounds chosen are recognized as possessing some weak anticonvulsant activity, there would appear to be no obvious correspondance with *in vitro* activity for those which have been compared pharmacologically (Schmitt & Schmitt, 1966).

The activity of dibenzepine compared to the imipramine-like tricyclic antidepressants is worthy of note. While no mention has been found of particular convulsant or anticonvulsant properties, dibenzepine has nevertheless a psychopharmacological profile which is distinctly different, especially with respect to its tranquillizing and anxiolytic character (Boissier, Simon & others, 1966). This activity, however, is in no way comparable to that of the benzodiazepines.

While these results do not disprove that there is a relation between the pharmacological and clinical potency of benzodiazepines and their interaction with the glycine receptor, this would nevertheless appear to be unlikely. A similar conclusion has been reported by Curtis, Game & Lodge (1976) who have shown that diazepam, at a pharmacologically active dose, does not significantly modify either the electrophysiological effect of strychnine with respect to glycine or its convulsant effect. The high *in vitro* concentrations involved compared with the low pharmacologically active doses have also been invoked as an argument against such a relationship (Costa, Guidotti & others, 1975) and if one accepts that the filtration technique gives a more reliable indication of an interaction with the glycine receptor, then these concentrations are even higher. The *in vitro* activities observed do not reflect large pharmacological differences and moreover the liposolubility of the benzodiazepines may well be an important factor in the displacement of [^{3}H]strychnine binding. That the tricyclic derivatives show effects comparable or superior to those of the benzodiazepines also argues against a specific interaction and, in any case, severely limits the possibilities of this *in vitro* system both as a criterion for benzodiazepine-like activity and as a predictive test for the selection of novel, potent anxiolytic drugs.

The capacity to distinguish glycine agonists from antagonists *in vitro* would greatly clarify the problem and as suggested by the activity of thiocolchicoside, should provide a means of detecting specific central myorelaxant or convulsant activity.

The specifically labelled [15-^{3}H]strychnine was prepared by Mr A. Jouquey, Radiochemical Laboratory. The R_m values were determined by Madame Dupuy, Physics Department.

The technical assistance of Mr C. Bonhomme and Mr J. C. Massardier and the helpful collaboration of Mr C. Marchandeau and Mr B. Willecomme are gratefully acknowledged. January 4, 1977

REFERENCES

BOISSIER, J.-R., SIMON, P., FICHELLE-PAGNY, J. & LWOFF, J.-M. (1966). *Thérapie*, **21**, 773–780.
COSTA, E., GUIDOTTI, A., MAO, C. C. & SURIA, A. (1975). *Life Sci.*, **17**, 167–186.
CURTIS, D. R., GAME, C. J. A. & LODGE, D. (1976). *Br. J. Pharmac.*, **56**, 307–311.
PLOTKA, C. & JEQUIER, R. (1957). *Archs int. Pharmacodyn. Thér.*, **109**, 386–399.
RANDALL, L. O. & KAPPELL, B. (1973). In: *The Benzodiazepines*, p. 27. Editors: Garattini, S., Mussini, E. & Randall. L. O., New York: Raven Press.
RUDZIK, A. D., HESTER, J. B., TANG, A. H., STRAW, R. N. & FRIIS, W. (1973). *Ibid.*, p. 285.
SCHMITT, H. & SCHMITT, H. (1966). *Thérapie*, **21**, 653–674.
YOUNG, A. B. & SNYDER, S. H. (1973). *Proc. Natn. Acad. Sci. U.S.A.*, **70**, 2832–2836.
YOUNG, A. B., ZUKIN, S. R. & SNYDER, S. H. (1974). *Ibid.*, **71**, 2246–2250.