J. Pharm. Pharmac., 1967, **19**, 240–245 Received October 28, 1966

The bioassay of γ-aminobutyric acid using a muscle preparation from *Ascaris lumbricoides*

A. S. F. ASH AND J. F. TUCKER

A method is described for the assay of γ-aminobutyric acid based on inhibition of the isolated dorsal muscle of *Ascaris lumbricoides*. The degree of relaxation produced by the acid was linearly related to the logarithm of the dose (0·5–2·0 μg/ml). Analyses of variance and indices of precision, calculated from the results of 6 model assays, indicated a satisfactory assay procedure. *Ascaris* was much more sensitive to γ-aminobutyric acid than to related amino-acids and was unaffected by dopa, dopamine, adrenaline, noradrenaline, 5-hydroxytryptamine or histamine (2 mg/ml) and by bradykinin (50 μg/ml).

THE distribution of γ-aminobutyric acid (GABA) and Factor I in biological tissues can be followed by measuring their inhibitory action on the impulses generated in the crayfish stretch receptor neuron (Florey, 1954; Elliot & Florey, 1956; Florey & Elliot, 1961; Levin, Lovell, Elliot & Elliot, 1961; Lovell, Elliot & Elliot, 1963). The crayfish hind gut, which is inhibited by GABA and related compounds, also provides a method of assay (Florey, 1961). Contractions of guinea-pig ileum induced by 5-hydroxytryptamine are antagonized by low concentrations of GABA (Hobbiger, 1958) but this preparation is affected by many endogenous substances.

An alternative preparation for the assay of GABA is now described using the isolated muscle of *Ascaris lumbricoides*, a round worm found in pig intestine. The method is more sensitive and specific than either of the crayfish preparations. A preliminary account of the work has been published (Ash & Tucker, 1966).

Methods

Preparation. Specimens of *Ascaris lumbricoides* were obtained fresh from the slaughterhouse and transported in a modified Tyrode solution, pH 6·5, at 37–38° (Goodwin, 1958). The composition of the medium was: NaCl 0·8, KCl 0·02, $CaCl_2$ 0·02, $MgCl_2$ 0·01, $NaHCO_3$ 0·015, Na_2HPO_4, $12H_2O$ 0·05, and glucose 0·5%. The medium was changed daily and the worms kept for not longer than 2 days at 37–38°.

The operative technique was similar to that described by Baldwin & Moyle (1947). A large active female worm was dropped from a height of about 12 inches on to the bench. This procedure stuns the worm and leads to a contraction of the musculature, which facilitates the dissection. The worm was secured, ventral surface upward, on a wax tray by passing two pins through the body, one in the region of the genital pore, the other a few mm from the head. The anterior and posterior portions of the worm were cut away and the remaining tissue was divided by a longitudinal incision in the ventral muscle. The divided edges were deflected and pinned down and the gut carefully removed with forceps. The two ventral muscle strips were then removed by cutting along the

From the Research Laboratories, May & Baker Ltd., Dagenham, Essex.

lateral canals. Ligatures were applied to the dorsal muscle which remained, one immediately in front of the genital pore, the other 2 to 2·5 cm further forward. Finally, tissue outside the ligatures was cut away. The isolated dorsal muscle strip was suspended in an organ bath of 10 ml capacity containing the modified Tyrode solution described above. The solution was gassed with ordinary commercial nitrogen which contained less than 0·5% oxygen. Movements of the tissue were recorded on a kymograph using a frontal writing lever with a magnification of ×20 and a load of 2 g.

Assay design. A four point design was chosen (Gaddum, 1953). To determine the precision of the assay, four known doses of GABA were used: two, A and B, were treated as "standard" and two, C and D, as "unknown". Doses were chosen, in the range 0·7 to 1·8 μg/ml, to give responses between 10 and 90% of the maximum, such that the ratio A/B = C/D = 2. The doses A, B, C and D were given in random sequence in each of 4 groups.

Results

RESPONSE OF ASCARIS MUSCLE TO GABA

GABA produced a rapid reversible inhibition of ascaris dorsal muscle in concentrations as low as 0·5 μg/ml. The inhibition, reflected as a relaxation of the preparation, was linearly related to the log dose (Fig. 1).

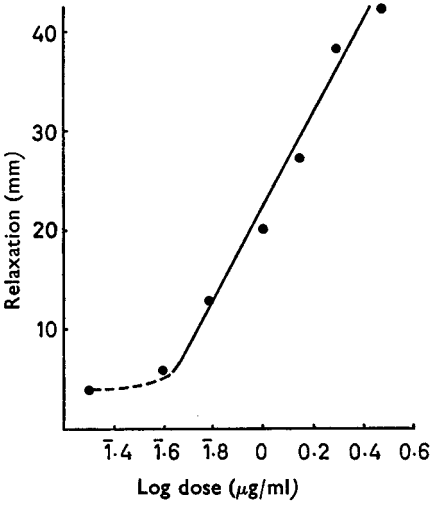

FIG. 1. Relaxation of isolated ascaris dorsal muscle in modified Tyrode solution by GABA, showing the linear relationship between dose and response which is regularly obtainable over this narrow range of doses.

Each administration of GABA was followed, after a latent period of approximately 15 sec, by a slow relaxation during a contact time of 3 min (Fig. 2). A dose cycle of 7 min allowed adequate recovery. The extent of the relaxation to any given dose tended to diminish during

the assay. This tachyphylaxis was unaffected by lengthening the interval between doses.

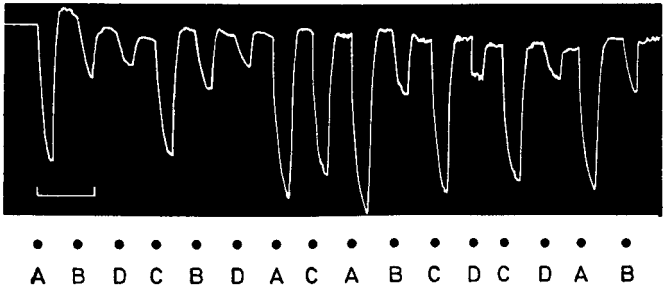

FIG. 2. Responses of isolated ascaris dorsal muscle to GABA in a model assay. Two doses of GABA, A and B (1·8 and 0·9 μg/ml), represent the "standard" and two doses, C and D (1·4 and 0·7 μg/ml), represent the "unknown". Doses were given in groups of 4, using a latin square design, allowing a contact time of 3 min and an interval of 7 min between doses. Time scale: 10 min.

ASSAY PERFORMANCE

The results of a typical assay, in which two solutions of known compositions were compared, are summarized in Table 1.

TABLE 1. INHIBITION OF ASCARIS MUSCLE BY FOUR DOSES OF GABA GIVEN IN FOUR SUCCESSIVE RANDOMIZED GROUPS

Dose (μg/ml)	Response (relaxation in mm) Groups 1	2	3	4	Sum
1·8	79	73	54	54	260
0·9	42	40	31	28	141
1·4	71	67	44	50	232
0·7	34	18	27	21	100
Sum	226	198	156	153	733

M, the logarithm of the ratio of potencies, is given by $M = (Yu - Ys)/b$, where Yu − Ys is the difference between the mean responses to unknown and standard, and b is the slope of the regression line (Schild, 1942). From the data in Table 1, M = 1·21. The estimated concentration of the "unknown", which had an actual concentration of 77·8 μg/ml, was then 82·7 μg/ml. The fiducial limits (95%) were 104·7 and 62·7 μg/ml.

TABLE 2. ANALYSIS OF VARIANCE OF GABA ASSAY. The probability (P) of the variations occurring by chance was calculated by means of the "F" test (Snedecor, 1956)

Source of variation	Sum of squares	Degrees of freedom	Variance estimate	F	P
Between groups	927·5	3	309·2	7·5	<0·01
Between standard and unknown ..	297·6	1	297·6	7·2	<0·05
Regression	3938	1	3938	949·5	<0·001
Deviation from parallelism	10·6	1	10·6	4	<0·05
Error	373·3	9	41·5		
Total	5547·0	15			

The analysis of variance (Snedecor, 1956) for this assay is summarized in Table 2. Differences between groups and between standard and unknown were significant; regressions were highly significant with negligible deviations from parallelism.

Precision of assay. The index of significance, more conveniently expressed as its reciprocal L, is a measure of assay precision. Typical values of L vary from 2 for an inaccurate assay to 30 for an accurate one (Gaddum, 1953). In 6 assays of GABA (Table 3) the mean value of L was 17.

TABLE 3. ASSAYS OF GABA ON ASCARIS MUSCLE. The precision of the assay is expressed by L, the reciprocal of the index of precision (Gaddum, 1953)

Experiment number	True value of unknown (μg/ml)	Estimated activity (μg/ml)	Fiducial limits (95%) of estimated activity (μg/ml)	L
1	77·8	73·0	54·1– 91·5	13·9
2	77·8	82·7	62·7–104·7	16·2
3	77·8	86·2	77·2– 96·4	30·0
4	77·8	78·0	50·6–106·9	12·5
5	77·8	74·7	57·5– 88·9	16·1
6	77·8	77·2	33·0–122·7	13·7

SENSITIVITY OF ASCARIS MUSCLE TO ENDOGENOUS AMINES AND ANALOGUES OF GABA

Ascaris muscle was stimulated by acetylcholine in doses as low as 0·5 μg/ml, but was unaffected by dopa, dopamine, adrenaline, noradrenaline, 5-hydroxytryptamine or histamine (2 mg/ml) and by bradykinin (50 μg/ml). In Table 4 the responses of the ascaris preparation to

TABLE 4. SENSITIVITY OF THREE PHARMACOLOGICAL PREPARATIONS TO GABA AND RELATED COMPOUNDS. Figures in parentheses are minimal effective concentrations. Data obtained in the present work* are compared with those calculated from values quoted in the literature (Bazemore, Elliot & Florey, 1956; McLennan, 1957, 1959; Robbins, 1959, Florey & Elliot, 1961†) and Florey, (1961‡).

Compound	Activity relative to GABA = 100		
	Inhibition of ascaris muscle*	Inhibition of impulse generation in the crayfish stretch receptor neuron†	Inhibition of acetylcholine induced contractions of the crayfish hind gut‡
γ-Aminobutyric acid	100 (0·5 μg/ml)	100 (1·5–10 μg/ml)	100 (2 μg/ml)
γ-Amino-β-hydroxybutyric acid ..	<0·001	50	10
Guanidinoacetic acid	43	150	200
Guanidinobutyric acid	7	33	(No action up to 100 μg/ml)
γ-Aminobutyrylcholine chloride hydrochloride	2·5	33	4
δ-Aminovaleric acid	9	5	(No data)
β-Alanine	1	5	2
ε-Aminocaproic acid	0·1	0·1	(No data)
Glutamic acid	<0·001	(Variable effects)	4

analogues of GABA are compared with values quoted in the literature for the two crayfish preparations. Five of the eight analogues of GABA are less active on the ascaris preparation than on the preparations of the crayfish.

Discussion

Studies of GABA have frequently been concerned with its possible physiological role, particularly in the central nervous system (for reviews see Elliot & Jasper, 1959; Curtis & Watkins, 1965; Elliot, 1965). There is, therefore, a need for assay preparations which are sensitive to GABA yet relatively unaffected by other common constituents in extracts of nervous tissue.

Both ascaris muscle and crayfish hind gut provide methods for the assay of GABA *in vitro* which are technically simpler than the original crayfish stretch receptor neuron preparation. The chief disadvantage of the ascaris preparation is that, unlike crayfish, it cannot readily be maintained for long periods in the laboratory. *Ascaris lumbricoides* can be kept alive for several days in suitable media at 37–38° (Baldwin & Moyle, 1947; Goodwin, 1958) but 48 hr outside the host is the limit if the worm is to be used for reliable assays of GABA. The use of commercial nitrogen to gas the bathing fluid during experiments with the preparation was introduced by Baldwin & Moyle (1947). High oxygen tensions are toxic (Laser, 1944), but it is still uncertain whether the worm is completely anaerobic (Fairbairn, 1957).

The basis of the assay is the linear relationship between the response of the muscle and the logarithm of the dose of GABA over the range 0·5–2·0 μg/ml. In the analysis of variance, the necessity for an assay design in which the effects of gradual changes in sensitivity are eliminated is illustrated by the variations between groups (Schild, 1942). The significant differences between standard and unknown, the highly significant regression and the negligible deviation from parallelism are indicative of a satisfactory assay procedure.

Ascaris lumbricoides dorsal muscle shows a greater specificity for GABA than the crayfish preparations, being less sensitive to related amino-acids. In fact, the contrasts in relative potency are such that parallel assays may be useful aids in the analysis of Factor I activity. So far there is no evidence that common endogenous amines will interfere with the use of the ascaris preparation for assay purposes.

References

Ash, A. S. F. & Tucker, J. F. (1966). *Nature, Lond.*, **209**, 306–307.
Baldwin, E. & Moyle, V. (1947). *J. exp. Biol.*, **23**, 277–291.
Bazemore, A. W., Elliott, K. A. C. & Florey, E. (1956). *Nature, Lond.*, **178**, 1052–1053.
Curtis, D. R. & Watkins, J. C. (1965). *Pharmac. Rev.*, **17**, 347–391.
Elliott, K. A. C. (1965). *Br. med. Bull.*, **21**, 70–75.
Elliott, K. A. C. & Florey, E. (1956). *J. Neurochem.*, **1**, 181–191.
Elliott, K. A. C. & Jasper, H. H. (1959). *Physiol. Rev.*, **39**, 385–406.
Fairbairn, D. (1957). *Expl Parasit.*, **6**, 491–554.
Florey, E. (1954). *Archs int. Physiol.*, **62**, 33–53.

Florey, E. (1961). *J. Physiol., Lond.*, **156**, 1–7.
Florey, E. & Elliott, K. A. C. (1961). *Meth. med. Res.*, **9**, 196–202.
Gaddum, J. H. (1953). *Pharmac. Rev.*, **5**, 87–134.
Goodwin, L. G. (1958). *Br. J. Pharmac. Chemother.*, **13**, 197–201.
Hobbiger, F. (1958). *J. Physiol., Lond.*, **144**, 349–360.
Laser, H. (1944). *Biochem. J.*, **38**, 333–338.
Levin, E., Lovell, R. A., Elliott, S. J. & Elliott, K. A. C. (1961). *J. Neurochem.*, **7**, 147–154.
Lovell, R. A., Elliott, S. J. & Elliott, K. A. C. (1963). *Ibid.*, **10**, 479–488.
McLennan, H. (1957). *J. Physiol., Lond.*, **139**, 79–86.
McLennan, H. (1959). *Ibid.*, **146**, 358–368.
Robbins, J. (1959). *Ibid.*, **148**, 39–50.
Schild, H. O. (1942). *Ibid.*, **101**, 115–130.
Snedecor, G. W. (1956). *Statistical methods Applied to Experiments in Agriculture and Biology*, 5th edn, p. 237, Ames, Iowa: Iowa State College Press.