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[^{3}H]γ-AMINOBUTYRIC ACID UPTAKE INTO NEUROGLIAL CELLS OF RAT SUPERIOR CERVICAL SYMPATHETIC GANGLIA

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SUMMARY

1. The influx of [^{3}H]γ-aminobutyric acid ([^{3}H]GABA) into isolated rat superior cervical ganglia has been measured by radioassay, supplemented by autoradiography. Ganglia were incubated in oxygenated Krebs solution at 25 °C, containing 10 μM-amino-oxyacetic acid. Under these conditions more than 95% of accumulated tritium was unmetabolized [^{3}H]GABA.

2. Ganglionic radioactivity increased linearly with incubation time, to yield an intracellular fluid/extracellular fluid concentration ratio (C_i/C_o) of about 200 after 6 hr in 0·5 μM-external [^{3}H]GABA.

3. Uptake showed saturation, with an apparent transport constant (K_T) of 6·8 μM and maximum influx velocity (J_i^{max}) of 7 μmole l. cell fluid^{-1} min^{-1}.

4. The influx rate at C_o = 0·5 μM was unaltered by raising *intra*cellular GABA from 0·2 to 1 mM.

5. Influx velocity increased with temperature (5–35 °C) in a monotonic manner with an apparent activation energy of 14 kcal mole^{-1}.

6. Concentrative uptake was depressed by reducing external [Na^+] with ouabain, by raising $[K^+]_o$ above 20 mM, or by removing external Cl^-. Uptake was not particularly sensitive to Ca^{2+} or Mg^{2+} ions.

7. Uptake of [^{3}H]GABA (0·5 μM) was inhibited by β-guanidinopropionic acid (apparent K_I, 28 μM), β-alanine (K_I, 55 μM), γ-amino-β-hydroxybutyric acid (K_I, 220 μM), β-amino-*n*-butyric acid (K_I, 708 μM), 3-aminopropanesulphonic acid (K_I, 832 μM) and taurine (K_I > 1 mM). Uptake was not depressed by 1 mM-glycine, α-alanine, leucine, serine, methionine or α-amino-*iso*-butyric acid.

8. Radioactively labelled methionine, leucine, glycine, serine, β-alanine and taurine (concentrations $\leqslant$ 5 μM) were also taken up by ganglia. Of these, only uptake of β-alanine and taurine were significantly depressed by 1 mM-GABA.

9. Autoradiographs confirmed that [^{3}H]GABA and [^{3}H]β-alanine were taken up predominantly into extraneuronal sites (presumed to be neuroglial cells). Methio-

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nine, leucine, glycine and serine showed preferential accumulation in neurones. Neuronal uptake of leucine was not prevented by inhibiting protein synthesis.

10. Calculations of net fluxes from unidirectional tracer fluxes suggest that the sympathetic glial cells are capable of promoting net uptake of GABA at external concentrations above 1 μM.

INTRODUCTION

The central inhibitory neurotransmitter γ-aminobutyric acid (GABA) is readily taken up by neuroglial cells in sympathetic ganglia, in a manner consistent with the operation of a high-affinity saturable membrane transport system (Bowery & Brown, 1972; Young, Brown, Kelly & Schon, 1973). This observation appeared rather anomalous at first, since GABA is not a transmitter in the mammalian peripheral nervous system. However studies on a number of other tissues have shown that transport systems for GABA are a rather common feature of neuroglial cells in both peripheral and central nervous systems (reviewed by Iversen & Kelly, 1975). Information regarding the glial carrier in ganglia might therefore be helpful in understanding this aspect of neuroglial function throughout the neuraxis.

With this in mind, we now present a more detailed account of our observations on the fluxes of radioactively labelled GABA in sympathetic glial cells than those published hitherto.

METHODS

All experiments were performed on superior cervical ganglia isolated from Wistar rats (either sex, about 250 g weight) under urethane anaesthesia (1·5 g kg^{-1} intraperitoneally injected). The connective tissue capsule surrounding the ganglion and its nerve trunks was removed (unless otherwise stated) and the ganglion was maintained in Krebs solution at 25 °C bubbled continuously with 95 % oxygen–5 % carbon dioxide gas mixture, to give a pH of 7·3–7·4. The composition of the Krebs solution, and variants thereof, is given below. Amino-oxyacetic acid (AOAA, 10 μM) was routinely added to all solutions to inhibit the metabolism of GABA by the ganglia (see Walsh, Bowery, Brown & Clark, 1974).

Uptake of radiolabelled amino acid

After pre-incubation in Krebs solution at 25 °C for at least 30 min following excision, radioactively labelled amino acid was added and the incubation continued for the desired time. The incubation temperature was 25 °C unless otherwise indicated. A volume of at least 0·5 ml. per ganglion was used (i.e. 500 times the ganglion volume), to prevent depletion of radioactivity from the medium. At the end of the incubation period the ganglion was rinsed briefly in non-radioactive solution, to wash off surface activity, blotted and weighed to within 10 μg (1 %) on an electro-torsion balance, using a time-extrapolation procedure to correct for drying (Brown, Halliwell & Scholfield, 1971). The ganglion was dissolved in 0·5 ml. Soluene (Packard), the mixture neutralized with 0·2 ml. 1·5 N-HCl, 10 ml scintillant added (Instagel, Packard) and radioactivity counted, together with triplicate aliquots of diluted incubation fluid, taken at the beginning and end of the incubation and added to the same scintillation mixture. Double-label (^{3}H + ^{14}C) radioassays were determined using the simultaneous equation method of Okita, Kabara, Richardson & LeRoy (1957).

Labelled substrates. A list of the radioactively labelled amino acids used in the present experiments is included in Table 1. Radiochemical purity of the parent material and stock incubation solutions made therefrom was assessed by subjecting 1 μc labelled material (with 10 μg added cold material) to thin-layer chromatography in ethanol (96 %)/ammonia (34 %) mixture (7 parts to 3 parts, by volume). Labelled material was located by contact autoradiography, and the unlabelled amino acid with ninhydrin (0·2 % in acetone). Autoradiographs revealed single radio-

active foci, with the exception of [^{3}H]methionine. Radiochromatogram scans or zonal radioassay of the silica indicated a radiochemical purity in excess of 90 % for the compounds used in the present study.

Metabolism. The chemical identity of radioactively labelled material accumulated by the ganglia was assessed in a series of preliminary experiments, in which a number of ganglia were incubated for prolonged (2–4 hr) periods in labelled amnio acid solutions, and then homogenized in 100 μl. ice-cold 0·4 M-perchloric acid solution. 10 μl. 3·3 M-K_2CO_3 in 0·5 M-triethanolamine hydrochloride was added, the homogenate centrifuged and a 10 μl. aliquot of the supernatant

TABLE 1. Proportions of parent labelled substrate present in ganglia incubated in labelled amino acid, determined by thin-layer chromatography

Amino acid	Label	Incubation: Time (min)	Incubation: Concn. (μM)	R_f unlabelled parent compound	% labelled material migrating as unlabelled parent
γ-Aminobutyric acid (GABA)	U-^{14}C	240	5	58	95†‡
β-Alanine	3-^{3}H	135	0·2	53	96
L-Serine	2-^{3}H	135	2	67	86
Glycine	2-^{3}H	135	1	58	93
Taurine	1,2-^{14}C	135	120	59	86
α-amino-*iso*-butyric acid	1-^{14}C	135	12·5	60	95
L-Leucine	4,5-^{3}H	120	0·2	76	40§
L-Methionine	me-^{3}H	120	1·4	62	21

† In the presence of AOAA; 63 % unchanged in absence of AOAA.
‡ Also estimated by high-voltage electrophoresis (see Walsh *et al.* 1974).
§ 46 % remained at the origin.

with added unlabelled parent material subjected to thin-layer chromatography as described above, using radioactively labelled parent material added to homogenates of unlabelled ganglia as controls. The proportions of accumulated radioactivity migrating with the parent compound are shown in Table 1: only [^{3}H]leucine and [^{3}H]methionine showed extensive metabolism as judged from the radiochromatographs. More detailed separation of radiolabelled GABA and metabolites, using thin-layer chromatography and high-voltage electrophoresis, indicated that more than 95 % of accumulated label corresponded to unchanged GABA when the incubation was undertaken in the presence of AOAA (Walsh *et al.* 1974; Bowery, Brown, Collins, Galvan, Marsh & Yamini, 1976). No corrections for metabolism were made in calculating total accumulation.

Protein synthesis. Incorporation of labelled amino acid into protein was measured separately by homogenizing ganglia in 10 % (w/v) trichloracetic acid (TCA), centrifuging at 50,000 *g* for 30 min, and measuring radioactivity separately in the supernatant and in the washed protein pellet. Experiments were replicated with and without cycloheximide (20 μg ml^{-1}) in the incubation solution.

Calculation of uptake. Tissue concentrations of radioactivity (measured as nc (g wet weight)$^{-1}$) were converted to concentration in the total intracellular fluid space by correcting for dry weight and extracellular (mannitol) space using previous data (Brown *et al.* 1971). Thus, dry weight was taken as 17 % of fresh wet weight and extracellular space after 30 min incubation as 0·41 ml. g^{-1}. Intracellular fluid was taken to be constant at 0·43 ml. g^{-1}, irrespective of incubation time. *Intracellular concentration* (C_i) was expressed as μmole *labelled* amino acid per l. cell fluid, by reference to the specific activity of the material in the incubation medium. The *intracellular/extracellular concentration ratio* (R) was calculated as the ratio of the concentrations of

radioactivity in the intra- and extracellular fluids (nc per l. cell fluid/nc per l. incubation medium).

Sources of variation in R. The concentration ratio R for [^{3}H]GABA attained under the same conditions of external substrate concentration and incubation time varied appreciably between different ganglia. Thus, a sample of twenty-three ganglia incubated for 30 min in 0·5 μm [^{3}H]GABA showed a coefficient of variance (c.v.) of 17·2% in R. The c.v. for R measured at different ^{3}[H]GABA concentrations covering a range from 10 to 90% of carrier-saturation (see Results) was approximately constant, suggesting that the variance arose from variations in factors determining maximal transport rate rather than from variations in carrier affinity. Such factors might be anatomical (e.g. surface area/volume ratio, which would affect non-equilibrium intracellular concentration if the surface carrier density were constant, or relative glial/total intracellular fluid volume ratio), or biochemical (e.g. variable ionic driving gradients). A mechanism adopted to minimize variance, where practicable, was to compare uptake rates in paired (contralateral) ganglia from the same rat. Thus, in a restricted test on six pairs of ganglia there was no significant difference in uptake between left and right ganglia after 30 min incubation in 0·5 μM-[^{3}H]GABA. Observed values for R were (mean S.E. of mean): *left*, 23·74 ± 1·15; *right*, 24·07 ± 1·37; (*right–left*), +0·319 ± 0·782. The c.v. was reduced from 16·0% overall to 7·6% for the ratio of R_{left}/R_{right}.

Autoradiography

The location of accumulated label was visualized by light-microscopic autoradiography using the method described by Young *et al.* (1973). Briefly, after incubation in labelled amino acid, the ganglion was rinsed for 30 min in non-radioactive solution, fixed for 60 min in 2·5% glutaraldehyde in 0·1 M-phosphate buffer (pH 6·8), washed, dehydrated, embedded in paraffin wax, sectioned at 4 μm, mounted, and coated with liquid Ilford G5 nuclear emulsion. Glutaradehyde is an effective fixing agent for amino acids since it can form a double Schiff-base linkage between the amino acid and amino-groups on structural moieties (Peters & Ashley, 1969; Orkand & Kravitz, 1971). Nevertheless, retention of [^{3}H]GABA during glutaraldehyde fixation and throughout the subsequent stages of washing and dehydration is incomplete (Young *et al.* 1973; see also Iversen & Bloom, 1972), implying that cross-linking is only partial. Notwithstanding, the general distribution of residual label was found to replicate fairly well that of the initial (unbound) material as judged by comparison with autoradiographs of frozen, freeze-dried sections (Young *et al.* 1973). The degree of retention in glutaraldehyde and comparison with unfixed autoradiographs was not determined for other labelled amino acids used in the present study.

Solutions

The composition of the stock incubation solution (normal Krebs-Henseleit solution) was (mM): NaCl, 118; KCl, 4·8; $CaCl_2$, 2·52; $NaHCO_3$, 25; KH_2PO_4, 1·18; $MgSO_4.7H_2O$, 1·19; D-glucose, 11. Pertinent ionic concentrations are (m-equiv l.$^{-1}$): [Na^+], 143; [K^+], 5·9; [Cl^-], 128. Modified solutions were prepared as follows.

Na^+-free (Li^+) solution: NaCl replaced by LiCl and $NaHCO_3$ by 12·5 mM-Li_2CO_3 (143 mM-[Li^+], 0 mM-[Na^+]).

Na^+-free (Tris$^+$) solution: NaCl and $NaHCO_3$ replaced by 143 mM-*Tris* base (tris[hydroxymethyl]aminomethane) titrated to pH 7·4 with HCl (143 mM-[Tris$^+$], 128 mM-[Cl^-], 0 mM[Na^+]).

Low Cl^- (sulphate) solution: NaCl substituted with 56 mM-Na_2SO_4 and KCl substituted with 2·35 mM-K_2SO_4 (143 mM-[Na^+], 5·9 mM-[K^+], 58 mM-[SO_4^{2-}], 5 mM-[Cl^-]). The tonicity was adjusted with D-mannitol.

Low Cl^- (isethionate) solution: NaCl replaced with 118 mM-Na isethionate (Na hydroxyethanesulphonate) and KCl replaced with 4·7 mM-K isethionate (123 mM-ise$^-$, 5 mM-Cl^-).

High K^+ solution: KCl was added to K-free normal Krebs solution in which KH_2PO_4 was replaced with NaH_2PO_4.

High K^+ (low Na^+) solution: K isethionate (in equimolar proportions) or K_2SO_4 (in hemimolar proportions, with added isomolar D-mannitol) were substituted for NaCl, to give up to 124 mM-K, 25 mM-Na.

Materials

Labelled amino acids. 2,3-[^{3}H]γ-Aminobutyric acid (4-amino-*n*-butyric acid), 2 and 10 c m-mole^{-1} (New England Nuclear Corporation, NEN); [U-^{14}C]γ-aminobutyric acid, 204 mc m-mole^{-1} (Radiochemical Centre, RC); [3-^{3}H]β-alanine, 37 c m-mole^{-1} (NEN); [1-^{14}C]β-alanine, 4·3 mc m-mole^{-1} (NEN); [1-^{14}C]glycine, 56 mc m-mole^{-1} (RC); [2-^{3}H]glycine, 8·6 c m-mole^{-1} (NEN); [1-^{14}C]DL-glutamic acid, 25 mc m-mole^{-1} (RC); [1,2-^{14}C]taurine, 2·5 mc m-mole^{-1} (NEN); [1-^{14}C]α-amino-isobutyric acid, 58 mc m-mole^{-1} (RC); [4-^{14}C]DL-2,4-diamino-*n*-butyric acid, 11·6 mc m-mole^{-1} (Schwarz-Mann); [methyl-^{3}H]L-methionine, 6·3 c m-mole^{-1} (NEN); [4,5-^{3}H]L-leucine, 42·7 c m-mole^{-1} (NEN); [2-^{3}H]L-serine, 8.6 c m-mole^{-1} (NEN).

Chemicals. γ-Aminobutyric acid (4-amino-*n*-butyric acid, B.D.H.); β-alanine (B.D.H.); L-α-amino-*n*-butyric acid (Sigma); DL-β-amino-*n*-butyric acid (Sigma); γ-amino-valeric acid hydrochloride (Sigma); ε-amino-caproic acid (Sigma); DL-γ-amino-β-hydroxy-*n*-butyric acid (Sigma); 3-amino-propanesulphonic acid (K. & K. Laboratories); L-2,4-diamino-*n*-butyric acid hydrochloride (Sigma); L-glutamic acid, monosodium salt (Sigma); glycine (B.D.H.), taurine (B.D.H.), β-guanidinopropionic acid (Sigma); guanidinoacetic acid (Sigma); γ-guanidinobutyric acid (Sigma); L-methionine (Sigma); L-leucine (Roche); L-serine (Sigma); N-methyl-GABA was synthesized by Dr M. J. Pringle according to the method of McElwain & Vozza (1949).

RESULTS

Part *A*. Radioassay of amino acid influx

1. *Uptake of* [^{3}H]*GABA*

Uptake kinetics

Time course. Text-fig. 1 shows the way in which the amount of [^{3}H]GABA taken up into isolated desheathed ganglia increased with incubation time. Uptake was measured at two external GABA concentrations (C_0 = 0·5 and 1 mM), and was calculated as the intracellular/extracellular concentration ratio R (see Methods). Uptake was approximately linear for at least 2 hr, by which time the concentration ratio R attained values greater than unity at both external substrate concentrations.

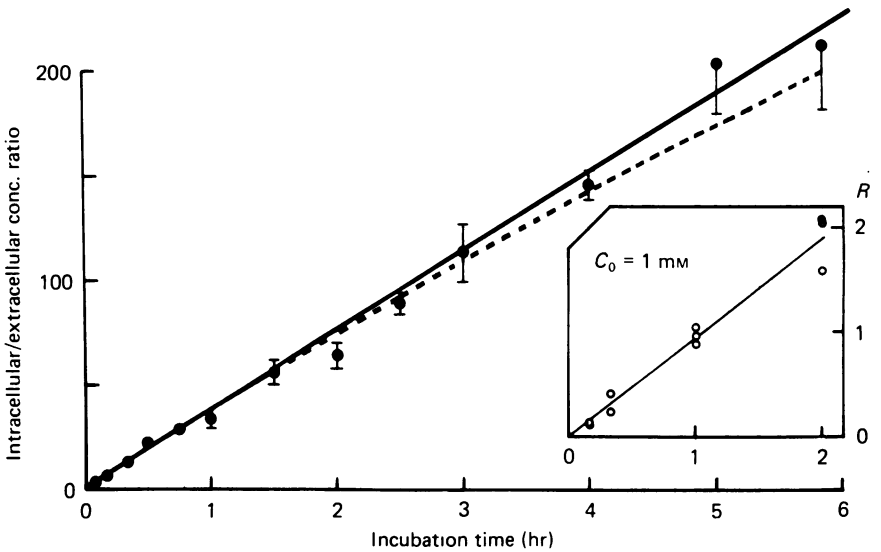
Accumulation of label might be expected to show an exponential approach to a steady-state value with a time-constant governed by the rate of backflux of label, such that:

$$R(t) = R(\infty)\,[1-\exp(-t/\tau)], \qquad (1)$$

where $R(t)$ is the concentration ratio at time t, R (∞) is the steady-state value at $t = \infty$ and $\tau = k_o^{-1}$, k_o being the rate coefficient for backflux. Linear uptake over long incubation times thus implies a very slow rate of backflux. This accords with previous measurements of the loss of [^{3}H]GABA from preloaded ganglia: the average efflux rate coefficient was 7×10^{-4} min^{-1} (giving a value for τ of around 24 hr), and was constant with varying internal GABA concentrations between 0·21 and 0·73 m-mole (kg. tissue)$^{-1}$ (Bowery *et al.* 1976). To illustrate the point, the interrupted line in Text-fig. 1 is drawn according to eqn. (1) with $\tau = 1{\cdot}4 \times 10^3$ min and assuming simple equilibration of [^{3}H]GABA with endogenous GABA, such that $R(\infty)$ = 914 (C_i = 0·457 mM: Bowery *et al.* 1976). The curve is indistinguishable from linear uptake, given the errors of experimental measurement. (In fact, uptake does not solely reflect equilibration since, at 1 mM-C_o, the final measured [^{3}H]GABA concentration in the tissue greatly exceeded the endogenous content: extrapolation through eqn. (1) suggests a steady-state value for C_i of about 30 mM at 1 mM-C_o.)

In ganglia where the outer connective tissue sheath was *not* removed the rate of uptake was 80% slower than that shown in Text-fig. 1. Conversely, the rate of uptake by desheathed ganglia was increased about 3 times by partly incising the ganglion at

several points with a razor blade, with the intention of improving access of label to the interior while preserving structural integrity. This suggests that the uptake rate was strongly influenced by the rate of inward diffusion: autoradiographic evidence for diffusional gradients is presented in Part *B*.



Text-fig. 1. Time-course for tritium uptake into isolated rat superior cervical ganglia after incubation in 0·5 μM-[^{3}H]GABA (C_o = 0·5 μM) and (inset) 1 mM-[^{3}H]GABA (C_o = 1 mM). Ordinates: intracellular/extracellular concentration ratio (R) for tritium (see Methods). Abscissa: incubation time (hr). Filled lines are the least-squares linear regression. The interrupted line is a calculated curve for an experimental approach to a steady-state value of R = 914 with rate coefficient corresponding to the experimentally observed efflux rate coefficient (k_o) of 7×10^{-4} min^{-1} (see eqn. (1)).

Concentration dependence. The average rate of uptake measured over 30 min incubation increased with external substrate concentration in the manner shown in Text-fig. 2. The influx rate measured up to 5 mM-GABA could be resolved into the sum of two parallel components, one saturable and one linearly dependent upon external substrate concentration. The total influx (J_i) may then be expressed as

$$J_i = \frac{J_i^{max} . C_o}{K_T + C_o} + k_i C_o, \qquad (2)$$

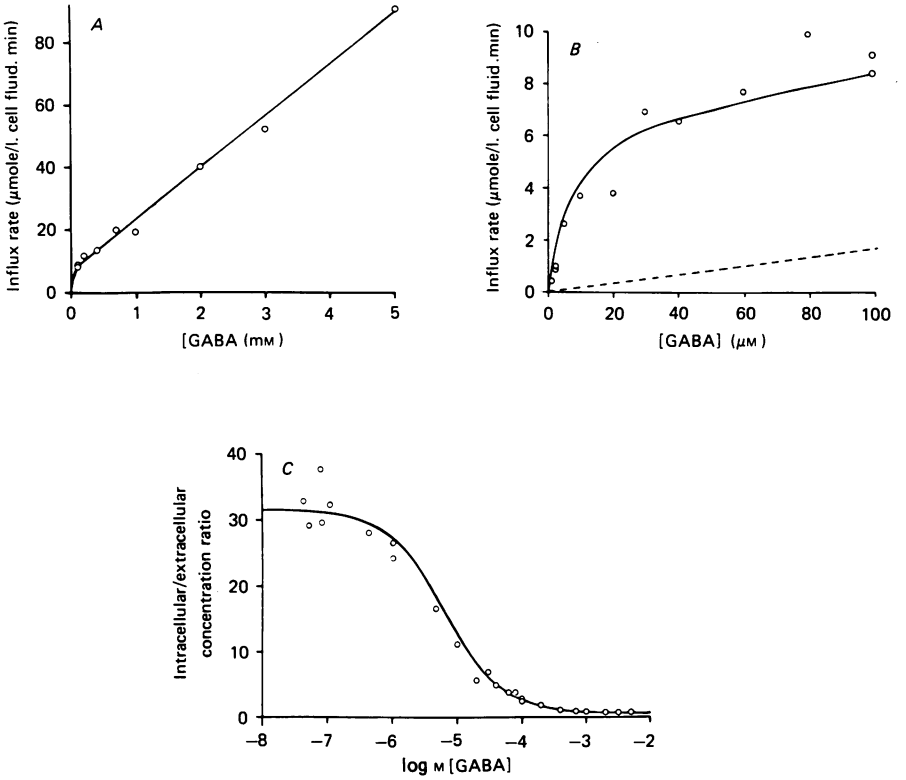
where C_o is the external substrate concentration and J_i^{max}, K_T are kinetic constants. Estimates for these constants were sought directly from the flux data using a programme for the minimization of the sums of residuals (Powell, 1968: Numerical Algorithms Group Programme EO4 4BF). The data was weighted on the basis of a constant coefficient of variance (see Methods), using Ottaway's (1973) method. Mean values (± S.E.) were

$$K_T = 6{\cdot}75 \pm 0{\cdot}49 \text{ μmole l.}^{-1};$$
$$J_i^{max} = 7{\cdot}01 \pm 0{\cdot}38 \text{ μmole (l. cell fluid)}^{-1} \text{ min}^{-1};$$
$$k_i = 1{\cdot}62 \pm 0{\cdot}11 \times 10^{-2} \text{ min}^{-1}$$

In Text-fig. 2C, the concentration-dependence of uptake is expressed in the form of an integrated rate-equation taking backflux into account (see Akedo & Christensen, 1962):

$$R(t) = k_o^{-1}[1-\exp(-k_o t)]\left[\frac{J_i^{max}}{K_T + C_o} + k_i\right], \quad (3)$$

where k_o is the rate coefficient for backflux and $t = 30$ min. The concentration ratio R then falls with increasing external substrate concentration C_o, with half-reduction at $C_o = K_T$: in effect, addition of unlabelled substrate produces auto-inhibition of the uptake of labelled substrate.



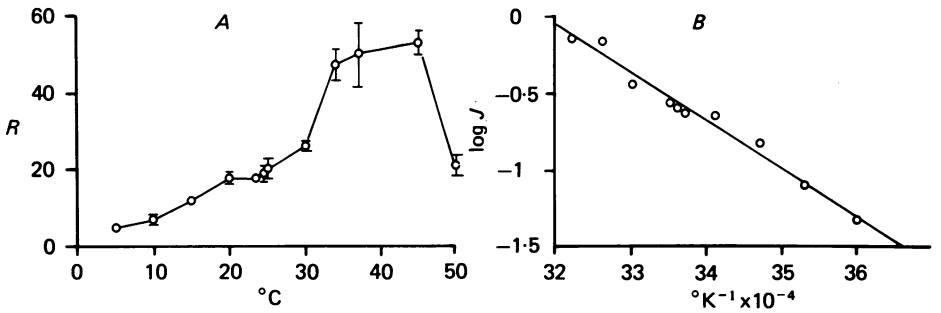
Text-fig. 2. Concentration-dependence of [^{3}H]GABA uptake measured after 30 min incubation. Graphs *A* and *B* show uptake expressed as average influx velocity (μmole/l. cell fluid. min), with backflux neglected. Curves show least-squares fits to eqn. (2) with $K_T = 6{\cdot}75$ μmole l^{-1}, $J_i^{max} = 7{\cdot}0$ μmole l^{-1} min^{-1} and $k_i = 1{\cdot}62 \times 10^{-2}$ min^{-1}. The dashed line shows the apparently non-saturable component of uptake. Graph *C* shows *total* uptake after 30 min, expressed as in Text-fig. 1, with $k_o = 7 \times 10^{-4}$ min^{-1} (see eqn. (3)).

Taking $k_o = 7 \times 10^{-4}$ min^{-1} (see above), the least-squares curve (drawn in Text-fig. 2C) yielded identical values for the kinetic constants to those deduced above assuming unidirectional influx; constants were not appreciably altered by a tenfold increase or decrease in the assumed value for k_o.

Effect of internal GABA concentration on [^{3}H]GABA influx

The saturable uptake component represented by eqn. (1) is essentially a binding term. It does not necessarily represent the binding preceding translocation: intracellular binding *following* entry could determine the rate of uptake. One way of

distinguishing these two possibilities is to saturate the supposed internal binding sites before adding labelled substrate (Heinz, 1954). In an experiment to test this, a number of ganglia were first exposed to 100 μM-[^{3}H]GABA for 4 hr, then rinsed for 30 min to remove 'unbound' label and finally incubated for a further period (30 or 240 min) in 0·5 μM-[^{14}C]GABA. At the end of the second incubation period the concentrations of ^{3}H and ^{14}C in the tissues were measured, and the amount of [^{14}C]GABA present compared with that in control ganglia not pre-incubated in [^{3}H]GABA. The concentration of [^{3}H]GABA in the ganglia after the initial incubation period was



Text-fig. 3. Effect of incubation temperature on the intracellular/extracellular concentration ratio (R) for [^{3}H]GABA measured after 30 min incubation in 0·5 μM[^{3}H] GABA. In A temperature is expressed in °C; each point is the mean of three or more ganglia (bars = S.E. of mean). Plot B is an Arrhenius plot of mean *log* influx rate (μmol (l. cell fluid)$^{-1}$ min^{-1}) *vs.* the reciprocal of the absolute temperature (in °K) for uptake data over the range 5–35 °C.

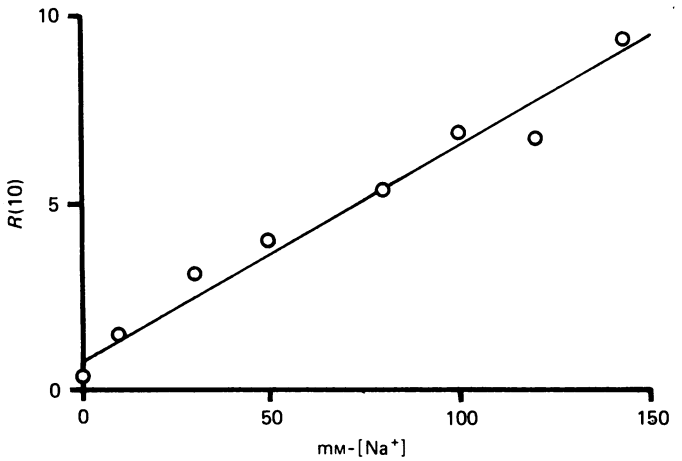
1·04 ± 0·03 mM (mean ± S.E., n = 6). Since the endogenous concentration of GABA in ganglia under these experimental conditions is 0·21 mM (Bowery *et al.* 1976), pre-incubation increased total intracellular GABA at least fivefold. Nevertheless, there was no change in the amount of [^{14}C]GABA subsequently accumulated. The mean ratio of ^{14}C uptake, preloaded or unloaded, in the six pairs of ganglia was 1·07 ± 0·08. (Individual values for this ratio were: 30 min pre-incubation, 0·85 and 1·00; 240 min, 1·02, 1·43, 1·16 and 0·94.)

Temperature

Uptake measured after 30 min incubation in 0·5 μM [^{3}H]GABA increased progressively as the incubation temperature was increased from 5 to 34 °C, then remained steady to 45 °C before declining (Text-fig. 3). The temperature optimum (37–45 °C) was thus rather higher than that previously reported for uptake by slices of brain tissue (Iversen & Neal, 1968). As suggested by the latter authors, the temperature optimum for net accumulation may be related more to the degree of metabolism and consequent rate of loss of radioactive metabolites than to any fundamental feature of the transport process *per se*: the higher optimum in the ganglion might then simply reflect the fact that metabolism was inhibited. An Arrhenius plot of *log* uptake against the reciprocal of the absolute temperature was linear between 5 and 34 °C (cf. ^{24}Na efflux: Brown & Scholfield, 1974*b*), the slope of which yielded an apparent activation energy of 14 kcal mole^{-1} (Text-fig. 3*B*).

Effect of ions

Extracellular Na. When extracellular Na^+ was replaced with $Tris^+$ the intracellular/extracellular concentration ratio R attained after 30 min incubation in 0·5 μM [^{3}H]GABA solution was reduced by 89·9 ± 0·7 % (mean ± S.E. of four pairs of



Text-fig. 4. Effect of replacing external Na^+ ions with equimolar concentrations of Li^+ on the intracellular/extracellular concentration ratio measured after 10 min incubation in 0·5 μM-[^{3}H]GABA. Each point represents a single determination. The Na^+ concentration was adjusted 15 min before beginning the incubation.

TABLE 2. Effect of reducing external [Na^+] on the uptake of [^{3}H]GABA. Paired (contralateral) ganglia were incubated for 10 min in [^{3}H]GABA (0·1 μM) with added unlabelled GABA (at the indicated concentration) in normal (143 mM-[Na^+]) solution or in a solution containing 70 or 25 mM-[Na^+]†. Intracellular/extracellular concentration ratios (R) in the two ganglia were compared, to give the ratio $R_{(Na=x)}/R_{(Na=143)}$. Each value represents a single (paired) comparison (ganglia incubated in low [Na^+] solution were pre-incubated in that solution for 10 min to ensure equilibration with interstitial fluid before adding GABA)

[GABA] (μM)	$\frac{R_{(Na=70)}}{R_{(Na=143)}}$	$\frac{R_{(Na=25)}}{R_{(Na=143)}}$
1	0·76	0·34
	0·77	0·35
10	0·72	0·33
	0·76	0·38
100	0·43	0·79
	0·56	0·35

† Na^+ replaced with Li^+.

contralateral ganglia incubated in Na^+ or $Tris^+$ solution). Replacement with Li^+ or K^+ produced a comparable inhibition of uptake. The *initial rate* of [^{3}H]GABA influx (measured after 10 min incubation) showed an approximately linear increase with increasing $[Na^+]_o$ (Text-fig. 4). In the converse situation, in which the concentration of GABA was varied at fixed values of $[Na^+]_o$, the fractional inhibition of [^{3}H]GABA uptake by a given reduction in $[Na^+]_o$ was independent of substrate concentration (Table 2). These effects of external [Na^+] might most easily be explained through a simple proportionality between the maximum saturable influx velocity (J_i^{max} in

eqn. (2)) and $[Na^+]_o$: it seems unlikely that external Na^+ depresses uptake by elevating the transport constant K_T, since the degree of inhibition should then diminish sharply with increasing substrate concentration. This accords with the principal effects of Na^+ on the influx of GABA into synaptosome preparations (Martin, 1973). It would seem that the translocational step, rather than the initial binding function, might be the major Na^+-dependent stage in the glial cell uptake process (see Stein, 1967; Schultz & Curran, 1970).

TABLE 3. Effect of ouabain (1 mM) on uptake of [^{3}H]GABA (0·5 μM, 10 min incubation starting 5 min after washing out the ouabain). Values are % inhibition of uptake compared with contralateral ganglia incubated without ouabain

Pre-incubation time in ouabain solution (min)	% inhibition of uptake	Increase in $[Na^+]_i$† (mM)
5	15	15
15	27	25
30	52	40

† Average increase in $[Na†]_i$ calculated from measurements of the rates of net ionic exchange in ouabain solution (Scholfield, 1975).

Intracellular Na: effects of ouabain. The average intracellular Na^+ concentration in isolated rat superior cervical ganglia incubated under the present experimental conditions is 22 mM as measured by flame photometry (Brown & Scholfield, 1974*a*) or 15 mM when calculated from ^{24}Na equilibration (Brown & Scholfield, 1974*b*). The concentration in the glial cells (the site of [^{3}H]GABA uptake (Young *et al.* 1973) may be slightly less than this (Brown & Shain, 1977). Total ganglionic Na^+ is rapidly increased by adding carbachol or by inhibiting the Na^+-pump with ouabain (Brown & Scholfield, 1974*a*); the effect of carbachol is very likely restricted to intraneuronal $[Na^+]$ (Brown & Scholfield, 1974*b*), whereas ouabain affects both neurones and glial cells (see Brown & Shain, 1977). In accordance with this, incubation in 550 μM-carbachol for 30 min, sufficient to produce a calculated *total* increment in intracellular $[Na^+]$ of 70 mM or more (and perhaps double this in the neurones) – did not reduce the concurrent uptake of [^{3}H]GABA. In contrast, ouabain clearly reduced uptake (Table 3). This may arise from three causes; elevation of intracellular $[Na^+]$; inhibition of Na/K-ATPase directly coupled to GABA transport; or a reduction in the transmembrane potential. Two factors point to an elevation of intracellular $[Na^+]$ as the primary cause. (i) The degree of inhibition of [^{3}H]GABA uptake increased with the length of pre-incubation in ouabain, in approximate proportion to the estimated increase in intracellular $[Na^+]$ (see Table 3). (ii) Appreciable inhibition of uptake persisted when the ouabain was washed out 5 min *before* adding [^{3}H]GABA. Inhibition of the Na-pump in the rat ganglion by ouabain is very rapidly reversible; moreover, during recovery from ouabain the membrane *hyper*polarizes through the electrogenic effect of the Na^+-pump (Brown, Brownstein & Scholfield, 1972).

Potassium. Elevation of extracellular $[K^+]$ from 6 to 124 mM by addition of KCl or K_2SO_4 (such that $[Na^+]_o$ was maintained at 143 mM) reduced the uptake of

[^{3}H]GABA (0·5 μM, 10 min) by 58 % on average. Substitution of K^+ for Na^+ produced much more inhibition (95 %).

Divalent cations. [^{3}H]GABA uptake was unaltered by omitting Ca^{2+} from the incubation medium or by raising $[Mg^{2+}]_o$ to 30 mM.

Cl. Replacement of external Cl^- (normally 118 mM) with sulphate or isethionate reduced [^{3}H]GABA uptake by about half.

TABLE 4. Effect of amino acids (1 mM) on simultaneous uptake of [^{3}H]GABA (0·5μM, 30 min incubation)

Amino acid	Concentration ratio (R) mean ± S.E.	(n)	% of control	IC_{50} (μM)
None	24·35 ± 0·64	(71)	100	
GABA	0·58 ± 0·39	(3)	2·4	6·7†
β-Guanidinopropionic acid	3·74 ± 0·54	(3)	15·4**	28
β-Alanine	5·81 ± 0·84	(3)	23·9**	55
γ-Amino-β-hydroxy-n-butyric acid	8·16 ± 1·28	(6)	33·5**	220
3-Aminopropanesulphonic acid	8·30 ± 1·68	(3)	34·1**	832
γ-Guanidinobutyric acid	8·49 ± 0·36	(4)	34·9**	—
N-Methyl-GABA	11·26 ± 0·75	(3)	46·2**	—
β-Amino-n-butyric acid	13·40 ± 0·98	(4)	55·0**	424‡
Guanidinoacetic acid	13·63 ± 2·49	(3)	56·0**	708
Taurine	14·30 ± 1·42	(3)	58·7*	> 1000
8-Amino-n-valeric acid	19·13 ± 1·49	(3)	78·6	
Glycine	19·21 ± 0·84	(3)	78·9	
L-2,4-Diamino-n-butyric acid	19·49 ± 2.27	(3)	80·0	
α-Amino-*iso*-butyric acid	21·63 ± 1·92	(3)	88·8	
α-Amino-n-butyric acid	21·98 ± 1·30	(3)	90·3	
L-Glutamic acid	22·77 ± 1·88	(3)	93·5	
ϵ-Amino-n-caproic acid	25·74 ± 2·58	(3)	105.7	
Serine	26·35 ± 4·02	(3)	108·2	
L-Methionine	27·51 ± 5·45	(3)	111·3	
L-α-Alanine	29·30 ± 4·87	(3)	120·3	
L-Leucine	30·23 ± 9·28	(3)	124·1	

* $P < 0·01$ (difference from control).
** $P < 0·001$.
† From Fig. 2.
‡ From Bowery *et al.* 1976.

Effect of amino acid analogues on [^{3}H]GABA influx

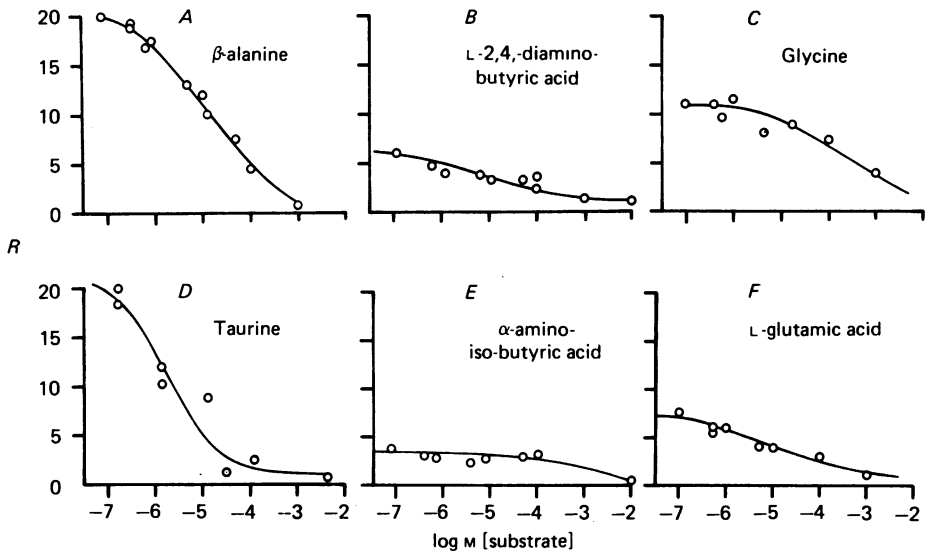
Table 4 shows the concentration ratio R attained after 30 min incubation in 0·5 μM-[^{3}H]GABA in the presence of a number of other amino acids at a concentration of 1 mM. Two points emerge. *First*, uptake was not inhibited by substrates for conventional neutral amino acid carriers (Christensen, 1975), such as glycine, serine, leucine, methionine, α-amino-*iso*butyric acid, α-amino-n-butyric acid or α-alanine. *Secondly*, however, uptake *was* strongly reduced by certain structurally related compounds, most notably, β-guanidino-propionic acid and β-alanine. Apparent inhibitor constants (K_I) for some of the more active compounds were deduced from inhibition curves at low substrate concentration (see fig. 1 of Bowery *et al.* 1976):

if inhibition is competitive, the concentration producing 50% inhibition (IC_{50}) approximates to K_I since [^{3}H]GABA $\ll K_T$.

In practice, the depression of uptake by these analogues did not accord fully with simple competitive inhibition, the slope of the inhibition curve being less than that expected (see Bowery *et al.* 1976). A more detailed analysis of the effect of β-alanine on [^{3}H]GABA influx revealed further deviation from simple competitive inhibition. For example, although the maximal influx velocity was not significantly altered, the apparent K_I increased with increasing inhibitor concentration.

2. *Uptake of other amino acids*

A number of other amino acids showed appreciable concentrative uptake ($R > 1$ after 30 min incubation) at low substrate concentrations (Table 5). The concentration dependence of some of these amino acids is shown in Text-fig. 5, by plots of the



Text-fig. 5. Plots of uptake of label *vs.* log external substrate concentration (see Fig. 2*C*) after incubating ganglia for 30 min in (*A*) [^{3}H]β-alanine, (*B*) [^{14}C]L-2,4-diaminobutyric acid, (*C*) [^{3}H]glycine, (*D*) [^{14}C]taurine, (*E*) [^{14}C]α-amino-*iso*-butyric acid and (*F*) [^{14}C]DL-glutamic acid. Curves are drawn by eye.

type illustrated in Text-fig. 2*C*. Uptake of L-2,4-diaminobutyric acid, α-amino-*iso*-butyric acid and DL-glutamic acid is rather low throughout the concentration range. Glycine uptake is more clearly saturable, but with a rather high half-saturation concentration (> 100 μM). Taurine and β-alanine show the closest resemblance to GABA. More detailed analysis of [^{3}H]β-alanine uptake by the curve-fitting procedure described above, and also by conventional double-reciprocal (Lineweaver–Burk) plots, suggested a K_T of between 50 and 75 μM (comparable to its K_I as an inhibitor of [^{3}H]GABA uptake, see Table 4) and a maximal influx velocity of about 12 μmole $l.^{-1}$ min^{-1}.

Uptake of both taurine and β-alanine was depressed by 1 mM-GABA. However, inhibition by GABA was not complete, even though the concentration used exceeded

TABLE 5. Effect of unlabelled amino acids (1 mM) on the uptake of radiolabelled substrates measured after 30 min incubation (R, mean ± S.E.; n = 3 unless otherwise indicated by figure in parentheses). Blank spaces: not determined

			Inhibitor						
Substrate	Concn. (μM)	None	GABA	Methionine	Leucine	Glycine	Serine	β-Alanine	Taurine
[^{3}H]GABA	0·25	23·2 ± 0·9 (23)	0·58[4] ± 0·04	27·5 ± 6·0	30·2 ± 9·3	19·2 ± 0·8	26·4 ± 4·0	5·81[4] ± 0·84	14·3[4] ± 1·4
[^{3}H]Methionine	0·35	26·9 ± 6·9	26·1 ± 10·5	3·03[1] ± 0·20	4·09[1] ± 0·29	13·9 ± 4·4	—	—	—
[^{3}H]L-Leucine	0·05	12·7 ± 1·7 (5)	16·7 ± 2·8	6·33[1] ± 0·77	2·14[4] ± 0·07	12·7 ± 1·9	—	9·7 ± 1·2	11·1 ± 0·5
[^{3}H]Glycine	0·1	11·0 ± 0·6 (12)	10·6 ± 0·8	—	8·44[2] ± 0·34	3·60[4] ± 0·22	6·35[4] ± 0·57	10·0 ± 0·6	—
[^{3}H]Serine	0·3	10·2 ± 0·7 (5)	12·5 ± 1·6	—	—	5·91[3] ± 0·34	6·12[2] ± 0·54	—	—
[^{3}H]β-Alanine	0·3	10·3 ± 0·5 (8)	2·72[4] ± 0·05	—	9·63 ± 0·11	9·37 ± 1·17	—	1·37[4] ± 0·03	5·33[4] ± 0·14
[^{14}C]Taurine	5	10·8 ± 0·5 (6)	7·6[1] ± 0·8	—	—	11·4 ± 0·7	—	1·58[4] ± 0·08	1·16[4] ± 0·10

[1] $P < 0·05$, [2] $P < 0·02$, [3] $P < 0·01$, [4] $P < 0·001$ (two-tailed t test, difference from control).

the GABA K_T by two orders of magnitude. In both cases auto-inhibition exceeded inhibition by GABA. Further, taurine uptake was inhibited more effectively by β-alanine than by GABA. This suggests the presence of a separate carrier for the shorter chain-length compounds taurine and β-alanine, which accounts for the major part of taurine uptake and a minor component of β-alanine uptake in this tissue. There was also separate cross-inhibition between glycine and serine and between leucine and methionine; none of these compounds were inhibited by GABA.

TABLE 6. Incorporation of label into TCA-precipitable fraction (see Methods). Ganglia were incubated in labelled amino-acid for 30 min, then washed for 30 min, in the absence or presence of 20 μg ml.$^{-1}$ cycloheximide. Numbers show (i) total uptake, supernatant plus precipitate (C_i/C_o) and (ii) % of total uptake in TCA-precipitate (Ppt); mean ± S.E. (n = 3 or 4 for each column)

Amino acid	Concn. (μM)	Control		+ Cycloheximide	
		C_i/C_o	Ppt (%)	C_i/C_o	Ppt (%)
[^{3}H]GABA	0·4	26·3 ± 3·1	0·9 ± 0·3	25·0 ± 1·0	1·5 ± 0·3
[^{3}H]β-Alanine	0·2	10·0 ± 0·8	1·6 ± 0·4	12·7 ± 0·6	1·9 ± 0·5
[^{3}H]Serine	0·5	7·1 ± 0·5	18·0 ± 1·2	6·4 ± 0·6	6·5 ± 0·4
[^{3}H]Glycine	0·5	6·0 ± 0·4	7·2 ± 0·3	5·0 ± 1·7	2·5 ± 0·5
[^{14}C]L-Leucine	0·5	9·3 ± 0·7	87·2 ± 1·1	7·7 ± 1·1	9·0 ± 0·7
[^{3}H]Methionine	0·3	0·70 ± 0·04	48·3 ± 0·3	0·86 ± 0·02	49·3 ± 1·2

A tentative classification of neutral amino acid carriers in the ganglion derived from Tables 4 and 5 might then be:

(*a*) a carrier for GABA (K_T 7 μM), with β-alanine (K_T 50–75 μM) and taurine (K_T > 1 mM) as alternative substrates;

(*b*) a separate taurine/β-alanine carrier;

(*c*) a carrier for glycine and serine; and

(*d*) a carrier for methionine and leucine.

Autoradiographic studies (see section *B* below) suggest that carrier *a* is restricted to glial cells, whereas carriers *b*, *c* and *d* are associated primarily with the neurones.

This classification is obviously very incomplete since it depends upon the inhibitory effects of a single (1 mM) concentration of competing substrate. Absence of cross-inhibition may therefore mean only that the transport constants for inhibitor and substrate are both in excess of 1 mM. Nevertheless, the distinction between the GABA carrier and other neutral amino acid carriers deduced earlier are clearly confirmed.

Incorporation of amino acids into protein. The amount of accumulated label incorporated into protein was estimated as the fraction of total label present in TCA-precipitated material after 30 min incubation (Table 6). Substantial amounts of leucine (87%), methionine (48%), serine (18%) and glycine (7%) were present in the precipitate. Cycloheximide greatly reduced the precipitate fraction of leucine, serine and glycine, but not that of methionine. However, cycloheximide had no effect on the *total* amount of amino acid (supernatant plus precipitate) taken up. Hence, protein incorporation or binding did not affect the influx rates, implying transport rather than intracellular binding to be the rate-limiting step. This accords with the observations for [^{3}H]GABA described above, in which the influx rate was independent of the intracellular GABA concentration.

Part *B*. Autoradiography

Autoradiographic localization of the [^{3}H]GABA accumulated by isolated ganglia has been described previously (Young *et al.* 1973). In brief, electron microscope autoradiographs showed that the uptake was substantially confined to neuroglial cells which are satellite cells around the neurones and Schwann cells around the nerve fibres (both pre- and post-ganglionic axons are predominantly unmyelinated in the

TABLE 7. Intraneuronal (N) and extraneuronal (E) grain counts in autoradiographs of ganglia incubated for 30 min in tritium-labelled amino acids at the concentration indicated. See text for details

(1)	(2)	(3)	(4)		(5)		(6)	(7)
Amino acid	Concentration (M)	No. of fields (n)	Total area ($\times 10^4$ μm^2) N	E	Total no. of grains N	E	Ratio of grain densities N/E (mean ± S.E.)	Fraction of neuronal grains
GABA	4×10^{-7}	9	1·56	5·86	383	5987	*0·24 ± 0·09*	*0·060*
	1×10^{-3}	5	1·47	2·65	79	598	*0·26 ± 0·06*	*0·117*
β-Alanine	3×10^{-7}	6	1·50	3·45	304	2218	*0·30 ± 0·04*	*0·121*
	1×10^{-3}	5	1·45	2·67	401	835	*0·88 ± 0·05*	*0·324*
Serine	3×10^{-7}	9	2·53	4·89	2696	2190	*2·32 ± 0·06*	*0·552*
Glycine	1×10^{-6}	9	2·78	4·64	2463	1676	*2·87 ± 0·44*	*0·595*
L-Leucine	3×10^{-7}	9	2·53	4·90	3801	2292	*3·39 ± 0·14*	*0·624*
Methionine	$2·6 \times 10^{-7}$	9	2·72	4·69	3727	1751	*4·07 ± 0·42*	*0·680*

rat: Foley & DuBois, 1945; Dunant, 1967). Glial uptake cannot be positively identified at the level of the light microscope in this tissue, for example, the satellite cell cytoplasm is < 1 μM deep over most of its area, but is reflected by an irregular distribution of silver grains in the spaces between the neurone somata, with a clear exclusion from the somata themselves (see Pl. 1). The primary aim of the present survey was to see how the distribution of some of the other amino acids accumulated by the ganglion compared with that of [^{3}H]GABA when viewed with the light microscope, and thence to deduce their approximate preferences for neurones or glia.

Examples of some autoradiographs, prepared after glutaraldehyde fixation as described in Methods, are shown in Pl. 1. Visually, two general patterns of distribution are apparent. (1) The location of silver grains following incubation in [^{3}H]GABA (Pl. 1*a* and *b*) and [^{3}H]β-alanine (Pl. 1*c* and *d*) was predominantly *outside* the neurone somata. The boundaries between low somatic and high extrasomatic activity were quite sharply drawn at both low (1 μM) and high (1 mM) concentrations of [^{3}H]GABA, and at low (0·3 μM) concentration of [^{3}H]β-alanine, but was rather blurred at 1 mM-[^{3}H]β-alanine. (2) In contrast, tritium-labelled methionine, leucine, serine and glycine, even at very low concentrations (< 1 μM), appeared preferentially accumulated in the neurone somata (exemplified in Pl. 1*e* and *f* by [^{3}H]glycine and [^{3}H]leucine respectively). After inhibiting protein synthesis [^{3}H]leucine still showed preferential retention in neurones.

Visual impressions of autoradiographs can be rather misleading. An attempt was made to quantify the distribution patterns by measuring the intrasomatic and extra-

somatic grain densities. Randomly selected fields from several sections were photographed ($\times 320$) and the transparencies projected onto paper (final magnification $\times 2144$). The neurone somata were outlined and individual silver grains marked and counted. The area of the somata and the residual (extrasomatic) spaces was determined by planimetry. Grain densities for each field were calculated after subtracting background densities measured over section-free areas of the emulsion. Autoradiographic exposure times were chosen to give not too wide a range of overall grain densities, to minimize variations in the degree of saturation of the emulsion. Total radioactivity was measured in contralateral ganglia incubated at the same time. The results of these measurements (Table 7) broadly accord with the visual impression: [^{3}H]GABA and [^{3}H]β-alanine gave intra- or extra-somatic density ratios $\ll 1$, whereas the other amino acids yielded density ratios in the range 2–4. Since the majority of developed silver grains arise from emission from the first μm layer of tissue (Oja, Oja & Hasan, 1967), the proportion of radioactivity within the tissue as a whole which lies within the somata is approximately indicated by the fraction of silver grains in the sections which overly the somata. This ranged from 6% with [^{3}H]GABA to 68% with [^{3}H]methionine. The residual material in the neuropil cannot be so subdivided into fibre and Schwann cell locations. However, if one assumes that exclusion from somata reflects an equal exclusion from fibres, then not less than 90% of the [^{3}H]GABA and (at low concentrations) [^{3}H]β-alanine would appear to accumulate in neuroglial cells. The same reasoning would lead to the converse conclusion, that the other amino acids are taken up predominantly by neurones and nerve fibres.

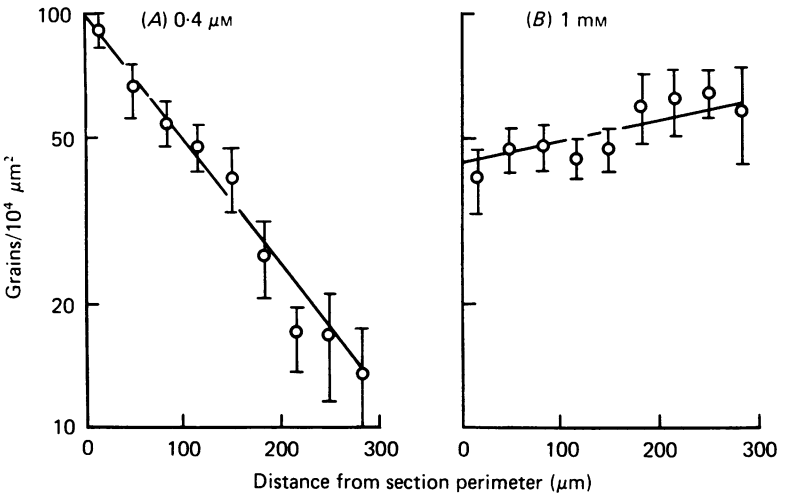
The following points are pertinent to the interpretation of these results. (*a*) The maximum pathlength of tritium β-radiation in materials of density around 1 is 3 μm, and the modal path length 1 μm. Hence, there is little possibility that silver grains over the central region of the neurones (mean diameter 24 μm (Brown & Scholfield, 1974*b*)) can derive from radioactivity outside the soma or beneath it. However, radiation from the satellite cells could lead to the appearance of silver grains up to 3 μm within the somatic boundary: this might account for some of the apparently-somatic radioactivity observed with GABA or β-alanine. (*b*) Since a silver halide crystal (average diameter 0·25 μm) receiving multiple 'hits' will register only one disintegration, saturation will tend to reduce the true grain count over strongly radioactive loci, and thus diminish the difference in grain density between high and low density areas. (*c*) The rationale behind using glutaraldehyde is to cross-link amino acids to structural proteins through Schiff base formation (Peters & Ashley, 1969). This process is incomplete, in the sense that a substantial proportion of the [^{3}H]GABA initially accumulated is lost from the tissue during fixation and the subsequent 'wet' procedures (Iversen & Bloom, 1972; Young *et al.* 1973). We have not directly measured the degree to which the other amino acids are lost, but comparisons of the observed grain density with the radioactivity in the contralateral ganglia did not suggest any radical difference from [^{3}H]GABA with respect to fractional retention. (*d*) The fact that an amino acid is 'fixed' by glutaraldehyde does not mean that it is fixed with equal ease at all sites, nor that it is fixed at its original location. Comparison with unfixed freeze-dried sections yielded a fair match with respect to [^{3}H]GABA location (Young *et al.* 1973), suggesting that the gross distribution pattern is not too seriously distorted by translocation or selective loss: nevertheless, such factors could affect quantitation.

Density gradients

In some of these autoradiographs, the grain density diminished appreciably on traversing the section from the perimeter of the ganglion to the centre. This transverse gradient was particularly sharp in ganglia which had been incubated in a low

concentration (< 1 μM) of [^{3}H]GABA or [^{3}H]β-alanine (Pl. 1*a* and *c*); the gradient was clearly less when a high concentration (1 mM) of these substrates was used (Pl. 1*b* and *d*).

A quantitative estimate of the transverse gradient obtained after incubating ganglia for 30 min in 0·4 μM and 1 mM-[^{3}H]GABA is shown in Text-fig. 6. The sections



Text-fig. 6. Distribution of silver grain densities observed on traversing ganglion sections from the periphery towards the centre measured at 33 μm intervals (see text). Ganglia were incubated in 0·4 μM (*A*) or 1 mM (*B*) [^{3}H]GABA. Ordinates: average grain density over each 30 μm band (grains per 10^4 μm^2 – means ± S.E. of nine sections in *A*, five sections in *B*). Abscissae: distance from outer perimeter of section (μm).

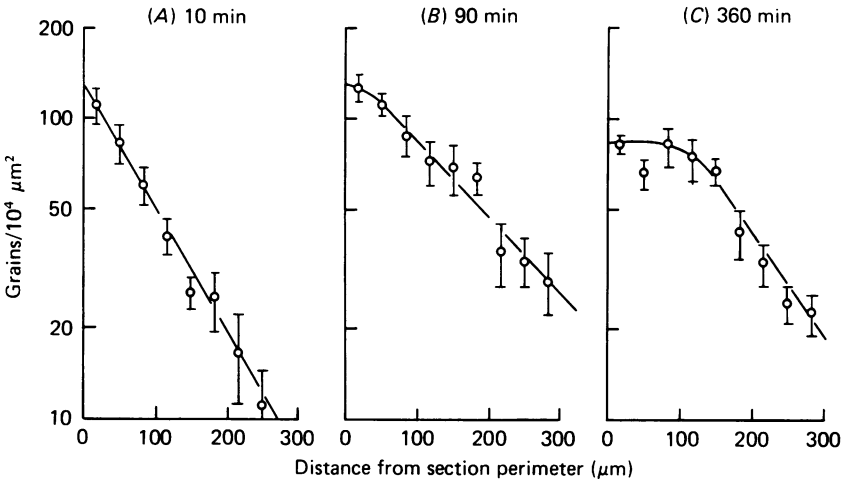
were exposed to emulsion for times selected to provide not too dissimilar peak densities, to minimize 'saturation' effects, and the average grain densities were measured in serial 30 μm bands from the perimeter of the sections toward the centre: the total distance traversed in this fashion ($\sim$ 300 μm radially) was set to equal the radius of the smallest section of the series. At 0·4 μM-[^{3}H]GABA, the grain density diminished in an approximately exponential manner with distance, giving an apparent 'space constant' of 130 μm. At 1 mM-[^{3}H]GABA there was no significant change in grain density: if anything the trend was toward an increased density on approaching the centre.

The effect of increasing substrate concentration was partly replicated by increasing the duration of incubation in [^{3}H]GABA (Text-fig. 7). However, the gradient was not entirely eradicated after incubating the tissue for 6 hr in 0·4 μM-[^{3}H]GABA.

The simplest explanation for these gradients is that, at low external substrate concentrations, the rate of uptake by glial cells at the periphery of the tissue is sufficiently rapid compared with the rate of inward diffusion to deplete the interstitial fluid of substrate. In consequence, access of substrate to deeper-lying cells is impeded. Depletion would be less pronounced at high substrate concentrations because, although the *absolute* rate of uptake is greater, the *proportion* of extracellular substrate taken up is less. Independent evidence for such a depleting effect of glial transport on interstitial GABA concentrations has been obtained previously by using the neuronal response as an index of interstitial GABA concentrations (Brown & Galvan, 1977).

Alternative explanations for the density gradient might be (*a*) that uptake in the centre of the

tissue is reduced through partial anoxia or (*b*) that glutaraldehyde penetration is too slow to prevent partial loss of radioactivity. Neither explanation seems plausible. The low average intracellular Na^+ concentration in whole ganglia (about 22 mM: Brown & Scholfield, 1974*a*) does not allow much margin for an appreciably anoxic core, nor would anoxia accord with the partial dissipation of gradients with increasing incubation time (when anoxia would be greater). Like-



Text-fig. 7. Grain density distribution in sections of ganglia incubated for (*A*) 10 min, (*B*) 90 min and (*C*) 360 min in 0·4 μM-[^{3}H]GABA, determined and plotted as in Text-fig. 6.

wise, uneven fixation by glutaraldehyde would not be expected to vary with incubation time or substrate concentration, particularly since the total (i.e. endogenous) GABA levels are not materially altered by the uptake of low concentrations of labelled GABA. Slow diffusion *per se*, unaffected by uptake, would not explain the gradients since the interstitial fluid is fully infiltrated by inert marker substances in 5–30 min (Brown *et al.* 1971). Autoradiographs made by the freeze-dried frozen section method (see Brown, Stumpf & Roth, 1969) showed full penetration of [^{3}H]inulin throughout the much larger cat superior cervical ganglion after 30 min incubation *in vitro* (Young *et al.* 1973 and unpublished observations).

DISCUSSION

In confirmation of previous experiments (Bowery & Brown, 1972; Young *et al.* 1973), the present experiments show the presence of a Na^+-dependent saturable uptake process for GABA in isolated rat superior cervical ganglia, localized to neuroglial cells. This process can be distinguished from that for other neutral amino acids in terms of both substrate-specificity and location. Thus, uptake of [^{3}H]GABA is unaffected by glycine, α-alanine, leucine, methionine or α-amino-*iso*-butyric acid. Further, although separate carriers for glycine-serine and leucine-methionine may exist, these appear to be associated more with neurones than glial cells. The GABA transport process in sympathetic glial cells also differs from that identified in nerve terminals from the brain (cf. Iversen & Johnston, 1971; Simon & Martin, 1973) in its greater resistance to inhibition by L-2,4-diaminobutyric acid and its greater sensitivity to β-alanine. This, and other, distinctions between neuronal and glial transport processes for GABA have been reviewed by Iversen & Kelly (1975).

Transport kinetics

Total tissue uptake rate could be described operationally as the sum of a saturable process, with an apparent transport constant (K_T) of about 7 μM, and a linear process showing no clear saturation up to 1 mM external substrate concentration. The former predominated at low substrate concentrations: for instance, it accounted for 80% of total uptake at 100 μM-GABA, 96·3% at 10 μM and 98·2% at 1 μM. However, it is quite uncertain how far this operational description of uptake by the ganglion as a whole might relate to transport kinetics at the cellular level, since several lines of evidence pointed to a severe diffusional restriction on measured uptake rates at low substrate concentrations. By analogy with unstirred layer effects (cf. Winne, 1973; Green, 1976), the principal effect of such diffusional restriction would be to cause an over-estimate of K_T; the maximal influx rates measured by extrapolation from measured fluxes at low substrate concentrates would be also underestimated, but that measured directly at carrier-saturating substrate concentrations would not. The complexity of the tissue precludes any realistic allowance for diffusional restrictions. It may, however, be relevant that a much lower value for K_T (0·1 μM) has been obtained for [^{3}H]GABA uptake by monolayer cultures of fetal rat sympathetic glial cells (Martin, Brown & Shain, 1976), in which diffusional restrictions are much reduced. It may further be questioned whether the apparent unsaturable component of total uptake truly reflects a separate membrane transport process, especially since the anatomical locus of uptake at high and low substrate concentrations appeared similar.

To some extent, diffusional restrictions also affect previously measured efflux rates: nevertheless, even when re-uptake was inhibited, the efflux rate coefficient remained low ($2{\cdot}4 \times 10^{-3}$ min^{-1}: Bowery *et al.* 1976). This slow back-flux can account for the long time-constant for [^{3}H]GABA uptake by ganglia. It is also instrumental in allowing the relatively slow inward transport process to generate very high intracellular–extracellular concentration gradients. Thus, at $\leqslant 1$ μM external GABA concentration, the steady-state concentration gradient across the glial cell membrane probably exceeds 10^3. As in nerve endings (Martin, 1973, 1976; Blaustein & King, 1976), the Na^+ gradient appears to be a determinant of concentrative uptake, to judge from the effects of remaining intracellular Na^+ or raising intracellular [Na^+] with ouabain.

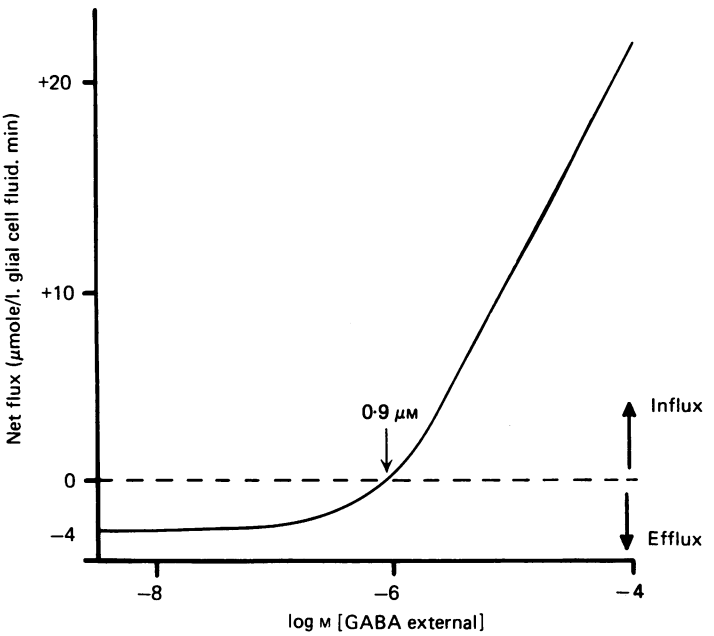
Net fluxes

Although net fluxes have not been measured directly in these experiments, they may be estimated from the observed unidirectional tracer fluxes, in combination with previous measurements of endogenous levels (Bowery *et al.* 1976):

$$\text{Net flux } J = \frac{J_i^{max} . C_o}{K_T + C_o} + k_i C_o - k_o C_i. \quad (4)$$

This presupposes that the unidirectional fluxes behave independently of each other. This seems to be the case, to the extent that they do not show accelerated exchange diffusion. Thus, tracer influx at low external substrate concentrations was not accelerated by raising internal GABA fivefold. Although tracer efflux *is* accel-

erated by high external GABA concentrations, this seems to result from reduced re-uptake of tracer rather than true exchange diffusion, since it is replicated by reducing external Na^+ (Bowery *et al.* 1976). Further, efflux of [^{3}H]GABA from monolayer cultures of sympathetic glial cells, where re-uptake is minimal, was not accelerated by external GABA (D. A. Brown & D. L. Martin, unpublished observations).



Text-fig. 8. Calculated initial net fluxes in ganglionic glial cells (in μmole GABA per l. glial cell fluid per min) at different external GABA concentrations. Calculations are based on measured tracer influx rates (this paper) and efflux rates measured in the absence of re-uptake (Bowery *et al.* 1976), and assume an initial intraglial concentration of 1·6 mM-GABA (Bowery *et al.* 1976).

Text-fig. 8 shows estimates of net fluxes expressed as changes in *intraglial* GABA concentration. This was calculated from tissue fluxes and concentrations assuming the glia to occupy about 13% of the total tissue volume or 30% of the total cell fluid (based on electron micrographs: see Bowery *et al.* 1976; the exact figure is not important to the form of the flux curve since the scaling factor is constant). The balance point at which total net fluxes are zero is critically dependent upon the rate of efflux (unlike the fluxes of labelled GABA). For this reason we have taken an efflux rate coefficient of $2{\cdot}4 \times 10^{-3}$ min^{-1} since this is the average value observed when re-uptake is totally inhibited (Bowery *et al.* 1976), and hence may approximate best to the true rate coefficient. As pointed out above, the estimates of kinetic constants may not be very accurate because of diffusional limitation. However, since the curve in Text-fig. 8 is based on *observed* fluxes (for which eqn. (4) can be regarded simply as an empirical description) influx and efflux constants would be affected by diffusion to similar extents.

The net flux calculations in Text-fig. 8 suggest that the fluxes are in balance at an external GABA concentration around 1 μM: at concentrations above this a net

uptake is expected (and indeed is readily demonstrable at concentrations of 100 μM or more), while a small net efflux might be anticipated below 1 μM. This accords closely with the conclusions of Sellstrom, Venema & Henn (1976) regarding the net fluxes of GABA in nerve endings from the brain.

Even though the flux data was derived from experiments in which metabolism of GABA (by transamination) was inhibited, this general conclusion remains valid for ganglia capable of transaminating GABA, since the effect of transamination is to increase the efflux of *metabolites* not of GABA itself. The calculations would be affected by transaminase inhibition only if this resulted in a change in the intracellular GABA concentration (in which case there would be a proportionate increase in the efflux rate, so shifting the balance point toward a higher external GABA concentration). Intraganglionic GABA concentrations are raised by transaminase inhibition *in vivo* (Bertilsson, Suria & Costa, 1976), but are not significantly altered *in vitro* in the absence of a continuous source of external GABA (Bowery *et al.* 1976), probably because the synthesis rate is very slow (Walsh *et al.* 1974; Kanazawa, Iversen & Kelly, 1976). In fact, transaminase-inhibition might be regarded as necessary *in vitro* to maintain endogenous GABA concentrations at their normal level: otherwise the leak of metabolites would rapidly lower the GABA levels.

The control of interstitial GABA concentrations

The calculations in Text-fig. 8 suggest that the glial transport system might drive the interstitial GABA concentrations toward a steady-state value of around 1 μM. However, they do not show how effective this process might be. Some information concerning this point has been derived from electrophysiological experiments in which the depolarization of the ganglionic neurones produced by GABA (see Adams & Brown, 1975) may be used to 'assay' interstitial (perineuronal) GABA concentrations. Changes in interstitial GABA concentrations resulting from activity of the glial cell transport system have been detected under two experimental conditions. First, when the uptake system was inhibited in the *absence* of external GABA, leaving a condition of unbalanced efflux, interstitial GABA concentrations showed a rise toward a steady-state value of 1–2 μM (Bowery *et al.* 1976). On the other hand, inhibition of uptake in the *presence* of external GABA suggested that glial transport reduced the interstitial concentration to a level well below that in the bathing fluid (for example, from 10 to 3 μM) within a period of 1–4 min (Brown & Galvan, 1977).

This latter effect may have appreciable bearing on the normal operation of the glial cell transport process, since the normal concentration of GABA in rat plasma is about 10 μM (Clark & Collins, 1976). Such a concentration applied directly to the neurones might well affect their excitability, whereas a concentration of 3 μM or less, though capable of producing a small depolarization, is below the threshold necessary to depress excitability (Adams & Brown, 1975 and unpublished observations). This suggests that the glial transport process normally exerts a protective action against excess extracellular GABA concentrations.

Other glial carriers

The GABA-carrier studied in these experiments seems to be very widely distributed in the mammalian peripheral nervous system. Thus, Schon & Kelly (1974*a*, *b*) have described a GABA-uptake mechanism in the satellite cells surrounding sensory ganglion cells, which seems virtually identical to that in the sympathetic ganglion

in terms of kinetics and substrate activity. Some of the GABA taken up by sympathetic ganglia enters the satellite cells, but not all: much also enters the Schwann cells around the unmyelinated fibres in the ganglion (Young *et al.* 1973). Further, peripheral nerve trunks also take up GABA, in approximate proportion to their content of unmyelinated nerve fibres (Bowery & Brown, 1972), in all probability into Schwann cells. Since mammalian sensory ganglion cells (De Groat, 1972; Feltz & Rasminsky, 1974; Deschenes, Feltz & Lamour, 1976; Gallagher, Higashi & Nishi, 1978) and peripheral unmyelinated fibres (Brown & Marsh, 1978) respond to GABA just like sympathetic ganglion cells, the above conclusions regarding the 'protective action' of the sympathetic glial carrier are probably applicable throughout the peripheral nervous system.

Glial uptake processes with similar characteristics are also present in the brain (see Iversen & Kelly, 1975). Inhibitions of glial uptake potentiates the effect of exogenous GABA on central neurones, but does not appear to prolong synaptic inhibition (Curtis, Game & Lodge, 1976; Scholfield, 1978; Lodge, Johnston & Curtis, 1978). Its function, then, might be to serve as a 'buffer' against extreme fluctuations in interstitial GABA concentrations over a wide area, rather than to terminate punctate transmitter action. The wide distribution of the carrier and its high capacity combined with relatively slow velocity, seem well suited to such a function.

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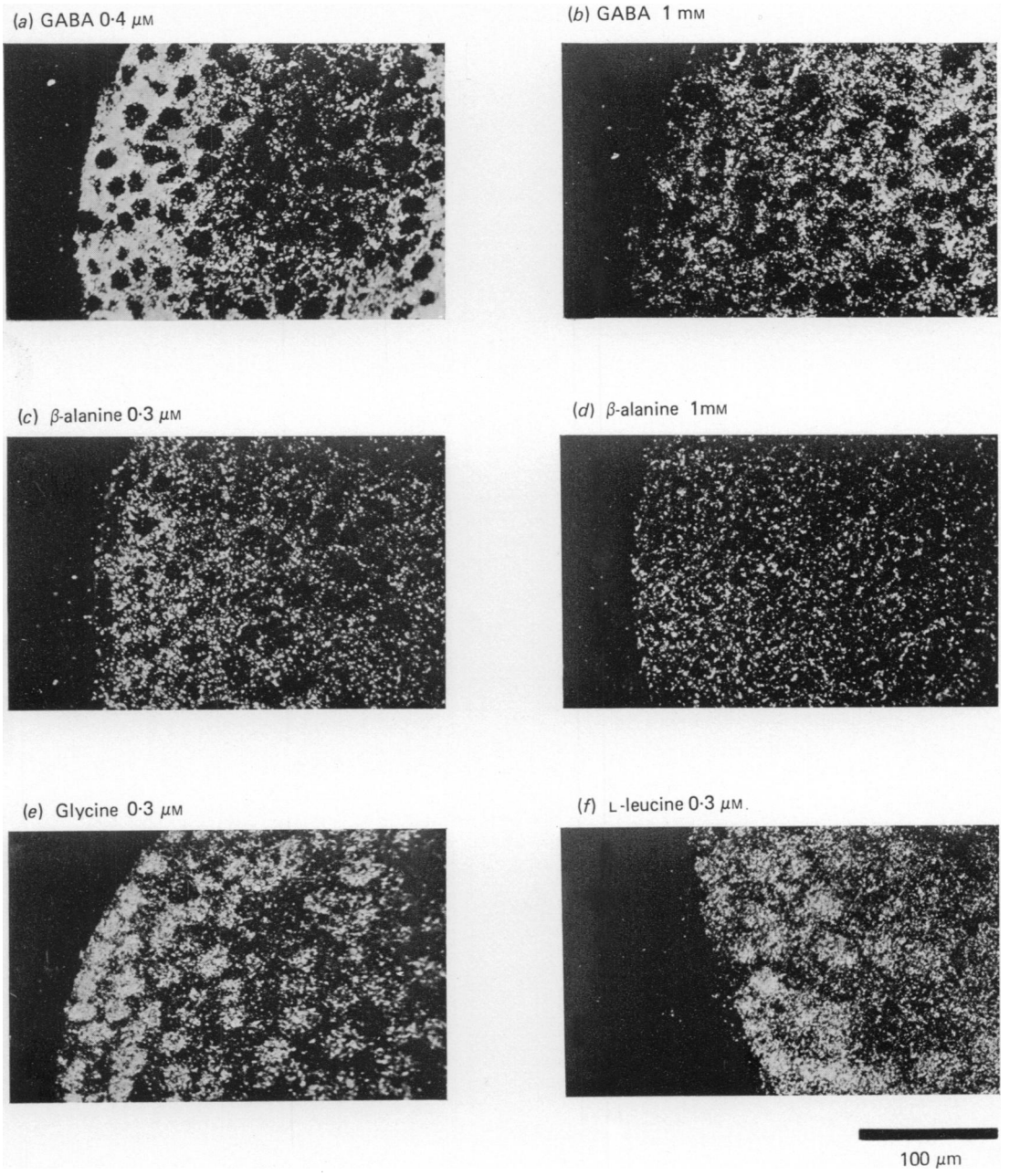
EXPLANATION OF PLATE

Autoradiographs of ganglia (radial sections) incubated for 30 min in tritium-labelled amino acids and viewed under reflected light. Autoradiographic exposure times were: *a*, 48 days; *b*, 48 days; *c*, 24 days; *d*, 50 days; *e*, 6 days; *f*, 3 days. The silver grains are white: black areas in *a*, *b*, *c* and white areas in *e*, *f* correspond to neurones.


(a) GABA 0·4 μM
(b) GABA 1 mM
(c) β-alanine 0·3 μM
(d) β-alanine 1mM
(e) Glycine 0·3 μM
(f) L-leucine 0·3 μM.
100 μm