The Distribution of Free Amino Acids in Subcellular Fractions of Guinea-Pig Brain

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1. The free amino acids of homogenates of guinea-pig brain in 0.32 M-sucrose and of subcellular fractions derived therefrom have been estimated by the method of Moore & Stein. 2. Seven amino acids together accounted for over 80% of the free amino compounds; these are, in decreasing order of abundance: glutamate, aspartate, γ -aminobutyrate, glycine, serine, alanine and threonine. In addition, there are appreciable quantities of amide (presumably glutamine). 3. Control experiments showed that the pattern of free amino acid occurrence in sucrose homogenates was similar to that of brains of animals killed by freezing in liquid nitrogen and extracted immediately without thawing. 4. The subcellular distribution of the amino acids resembled that of soluble cytoplasmic markers; there was no specific localization in a fraction rich in isolated presynaptic nerve terminals of amino acids capable of exciting or depressing central neurones. 5. The significance of the results is discussed in relation to the possible role of centrally active amino acids as transmitters.

Two amino acids present in uniquely high concentration in brain tissues, glutamate and γ -aminobutyrate, have attracted attention as possible synaptic transmitter substances because of their ability, in very small amounts, to stimulate or depress cortical and other central neurones. As little as 10^{-15} moles of glutamate, if applied iontophoretically through a micropipette in close proximity to a cortical neurone, will cause it to discharge a train of electrical impulses, and similar amounts of γ -aminobutyrate will depress neuronal activity (Krnjević & Phillis, 1963; Krnjević, 1965).

For this reason we have for some time been studying the subcellular distribution of glutamate, γ -aminobutyrate and other free amino acids of brain tissue by using an automatic amino acid analyser and subcellular fractionation techniques developed in this Laboratory for the study of bound acetylcholine and other putative central transmitters (reviewed by Whittaker, 1965). When brain tissue is homogenized under carefully controlled conditions, a considerable proportion of the nerve endings survive homogenization and are torn away from their attachments. The free nerve endings, for which the term 'synaptosomes' has been proposed (Whittaker, Michaelson & Kirkland, 1964), may be separated as a distinct fraction by differential and density-gradient centrifuging. This

fraction may be expected to be rich in transmitter substances; and it is, in fact, found to contain most of the bound acetylcholine, noradrenaline and hydroxytryptamine of the tissue. It also provides a starting point for the preparation of component structures of the nerve endings such as synaptic vesicles, external membranes, intraterminal mitochondria and neuronal cytoplasm. Other fractions obtained from the original homogenate contain nuclei, myelin fragments, mitochondria, microsomes and cell sap. The analysis of all these fractions can thus provide much information about the distribution of substances within morphologically defined subcellular compartments of nervous tissue.

A preliminary account of our work on amino acid distribution was given by Whittaker (1963). During the course of this work, column-chromatographic analyses of the free amino acids of bee and dog brain have been presented by Frontali (1962) and Tews, Carter, Roa & Stone (1963) respectively. Earlier work has been reviewed by the latter authors. The subcellular distribution of glutamate and γ -aminobutyrate in rat and mouse brain has been studied by Ryall (1964) and Weinstein, Roberts & Kakefuda (1963) by using fractionation procedures based on earlier work from this Laboratory (Hebb & Whittaker, 1958; Whittaker, 1959).

METHODS

Preparation of brain fractions

Two series of experiments were carried out, with whole brain and cortex respectively. The general conditions were similar to those described by Gray & Whittaker (1962), and were such as to provide a maximum yield of synaptosomes (Whittaker & Dove, 1965). Homogenization was carried out in a glass-and-Perspex homogenizer of the type described by Aldridge, Emery & Street (1960) with a clearance (on diameter) of 0.25 mm. and a rotational speed of 840 rev./min. All operations were carried out at 0–4° in a cold laboratory with glass-distilled water and analytical-grade reagents.

Primary fractions. Unless otherwise stated, three or four guinea-pig brains (the whole brain rostral to the superior colliculi; total weight 8–12 g.) were homogenized in ice-cold 0.32 M-sucrose to give a final tissue concentration of 10% (w/v). In some experiments the homogenate was centrifuged immediately, or at various times after preparation, at 100 000g for 60 min. to give a total particulate preparation (*P*) and a high-speed supernatant (*S*). In other experiments it was separated into nuclear (*P*₁), crude mitochondrial (*P*₂), microsomal (*P*₃) and supernatant (*S*₃) fractions as described by Gray & Whittaker (1962). The particulate fractions were sedimented by centrifuging the homogenate, or supernatant from the previous spin, at 1000g for 11 min. (*P*₁ fraction), 17 600g for 60 min. (*P*₂ fraction) and 100 000g for 60 min. (*P*₃ fraction). The *P*₁ fraction was twice washed by resuspension in 0.32 M-sucrose and recentrifuging at 1000g for 11 min.; the washings were added to the initial supernatant before centrifuging the latter to give fraction *P*₂. Pellets were resuspended in 0.32 M-sucrose where necessary for analysis.

The *P*₂ fraction was separated into three subfractions containing mainly myelin (fraction *A*), synaptosomes (fraction *B*) and mitochondria (fraction *C*) by means of a two-step discontinuous density gradient made up of equal volumes of 0.8 M- and 1.2 M-sucrose as described by Gray & Whittaker (1962). Separation was achieved in the SW25 head of the Spinco model L preparative ultracentrifuge after 2 hr. at 53 500g. Fraction *A* was the material floating on 0.8 M-sucrose, fraction *B* that floating between 0.8 M- and 1.2 M-sucrose and fraction *C* that sedimenting below 1.2 M-sucrose.

For preparing subfractions of disrupted synaptosomes (see below) the *P*₁ fraction was not washed and the *P*₂ fraction was sedimented at 100 000g for 20 min. This diminished the yield of synaptosomes but speeded preparation and cut down microsomal contamination. Similar fractions were prepared from guinea-pig cortical tissue.

Subfractions of disrupted synaptosomes. Fractions containing the soluble cytoplasmic constituents of synaptosomes (fraction *O*), synaptic vesicles (fraction *D*), microsomes (fraction *E*), larger (external) membrane fragments (fractions *F* and *G*), incompletely disrupted synaptosomes (fraction *H*) and mitochondria (fraction *I*) were prepared from the supernatant (*W*₂) of a *P*₂ fraction from whole brain after resuspension in water and removal of larger fragments (mostly myelin fragments and large mitochondria) at 100 000g for 20 min. as described by Whittaker *et al.* (1964). Similar fractions were prepared from cortical synaptosomes by 'method B' of Eichberg, Whittaker & Dawson (1964) (see also Whittaker & Sheridan, 1965). In this procedure, the cortical *P*₂ fraction described above was washed by

resuspension in sucrose, resedimented at 100 000g for 30 min. and then disrupted by suspension in water (2 ml./g. of original tissue). The suspension was layered (5 ml./tube) on top of a continuous sucrose density gradient ranging from 0.4 M-sucrose at the top to 0.6 M-sucrose at the bottom (15 ml./tube), which had in turn been formed on top of a layer of 1.6 M-sucrose (5 ml./tube) in a Lusteroid tube of the Spinco SW25 head. After centrifuging at 53 500g for 2 hr., a clear top layer (*O*) containing soluble cytoplasmic components had formed above a hazy band (*D*) of synaptic vesicles; below this, floating between 0.6 M- and 1.6 M-sucrose, was a dense layer (*J*) containing partially disrupted synaptosomes, membrane fragments and mitochondria. This fraction corresponded in density and composition to fractions *E–I* of the whole-brain preparations. The tube was sliced to separate the clear, hazy and dense layers. In some experiments, the *D* layer was diluted with an equal volume of water and centrifuged at 100 000g for 60 min. to give an almost clear supernatant (*D*_s) and a pellet (*D*_p) of closely packed synaptic vesicles.

Frozen brain extracts. To compare the amino acid content of sucrose homogenates with that of brain extracts prepared under conditions in which post-mortem changes in the concentration of labile constituents are believed to be minimal, small guinea pigs (weighing less than 200 g.) were killed by immersion in liquid N₂ and the frozen brains removed from the skull by means of a cold chisel and bone forceps. The brain was quickly powdered while still frozen in a chilled mortar and extracted with ethanolic 12.5 M-NHCl (4 vol. diluted with 1 vol. of water; 100 ml. of diluted extractant/g. of tissue).

Extraction and analysis of amino acids

Amino acids were extracted from brain fractions by the addition of 4 vol. of ethanolic 12.5 M-NHCl. Particulate material, if still present, was removed by centrifuging the suspension at 100 000g for 20 min. and the supernatant (or original solution) reduced in volume *in vacuo* to about 10 ml. in a rotary evaporator at 35°. To avoid interference by sucrose and electrolytes in subsequent chromatography, the amino acids were adsorbed from the extract on to the acidic ion-exchange resin, Zeo-Karb 225 (15–30 mesh; The Permutit Co. Ltd., London) and eluted with aq. NH₃ as described below. The Zeo-Karb 225 was prepared for use by heating it (0.45 kg.) with three successive amounts (1 l.) of N-NaOH at 100° for 2 hr. The resin was then washed thoroughly with water, converted into the H⁺ form with N-HCl and finally again washed with water.

Adsorption and elution of amino acids from fractions. Two polythene bottles, each containing 4 g. of resin, were used for each extract. The resin samples were first washed by shaking with water, which was discarded. The fraction was poured into bottle 1 of the pair, shaken mechanically for 10 min., the resin allowed to settle and the supernatant transferred to bottle 2. An equal quantity of water was added to bottle 1. The bottles were then shaken for a further 10 min. The supernatant in bottle 2 was discarded, that from bottle 1 transferred to bottle 2 and a further quantity of water added to bottle 1. The shaking and transfer procedure was repeated. Finally, the supernatant from bottle 2 was again discarded and that of bottle 1 was transferred to bottle 2, which was shaken for 10 min., after which the supernatant was discarded.

The amino acids were eluted from the resin by means of

aq. 5N-NH₃ (10–20 ml.) added to each bottle. After shaking for 10 min. the supernatants were collected and elution was repeated with a further 10–20 ml. of aq. 5N-NH₃. The combined supernatants were reduced in volume on the rotary evaporator and made up to 5 ml. Samples were taken for amino acid analysis by column chromatography as described below. For total amino nitrogen estimations by the ninhydrin method the extracts were freed from any traces of NH₃ still present by adding an equal volume of 10% (w/v) potassium metaborate and storing them over conc. H₂SO₄ and P₂O₅ in a desiccator for 12–15 hr. under reduced pressure. They were then diluted 1:10 before analysis.

Ninhydrin-positive substances in column-chromatographic fractions were similarly extracted for subsequent paper chromatography, except that 40 g. quantities of resin were used per bottle.

Estimation of carboxyl amino nitrogen. This was carried out on the extracts manometrically by using the chloramine-T method of Gerok & Waller (1956).

Estimation of total amino nitrogen by ninhydrin. This was carried out essentially as described by Chibnall, Mangan & Rees (1958). Diluted extract (1 ml.), freshly prepared KCN-acetate buffer (made by mixing 23 ml. of 4M-sodium acetate buffer, pH 5.5, 75 ml. of 2-methoxyethanol and 2 ml. of 10 mM-KCN) (1 ml.) and 10% (w/v) ninhydrin (0.2 ml.) were heated at 100° for 15 min. in capped tubes, cooled and diluted to 10 ml. with ethanol, and the extinctions at 570 mμ compared with those of (a) standard solutions of glutamic acid containing 1–6 μg. of N/ml. and (b) a resin blank obtained by shaking resin with water and eluting any adsorbed substances with aq. NH₃. Since the extinctions of the ninhydrin complex with different amino compounds vary, the method cannot claim to be very accurate, but it was useful in deciding how much of a fraction was needed for chromatography and to get an approximate idea of how total amino nitrogen was distributed in the various fractions.

Analysis of constituent amino acids of extracts by column chromatography. The constituent amino acids of the extract were analysed by chromatography on jacketed columns of sulphonated polystyrene resin by using an automatic analyser to record the concentration of ninhydrin-positive material in the effluent. The analyser made use of a sampling and mixing device based on two motor-driven syringes. A complete analysis required about 7 μg. atoms of amino nitrogen. Initially, neutral and acidic amino acids and basic amino acids were analysed separately, the former by the two-buffer system of Spackman, Stein & Moore (1958) with a 1 cm. × 150 cm. column and 0.2M-sodium citrate buffer, pH 3.25, at 30° followed by 0.2M-sodium citrate buffer, pH 4.25, at 50°, and the latter on a 1 cm. × 50 cm. column with 0.38M-sodium citrate buffer, pH 4.26, at 30° and 50°. Later a simpler procedure was developed by using a 1.5 cm. × 60 cm. column of resin beads of uniform (18 μ, s.d. ± 4 μ) diameter (Hill & Mangan, 1964) and gradient elution. After an initial elution with 0.2M-sodium citrate buffer, pH 3.25, at 30°, elution with 0.2M-sodium citrate buffer, pH 4.25, at 50° was commenced and the ionic strength and pH of the eluting fluid were continuously raised by the addition of 0.33M-trisodium citrate to the buffer reservoir. The amount of each component was estimated by integration of the area under the peaks by using the width-and-height method of Spackman *et al.* (1958).

Table 1. *Recovery of amino acids from a mixture*

Amino acids in mixture	Concn. in mixture (μg. atoms of N/ml.)	Amount recovered (% of original amount)
Aspartate	1.22	112
Threonine	1.89	98
Serine	1.26	98
Glutamate	0.89	128
Glycine	3.37	98
Alanine	1.00	94
γ-Aminobutyrate	1.80	80
Lysine	1.79	100
Total	13.17	102
Total amino N (by ninhydrin)	10.17	77

Combined paper chromatography and paper electrophoresis of fractions separated by column chromatography. The compounds present in column-chromatographic peaks were adsorbed on to resin and eluted as described above. They were then submitted to combined paper chromatography and paper electrophoresis as described by Dawson, Hemington & Davenport (1963), by using chromatography in a phenol-acetic acid-ethanol-water mixture in one direction and ionophoresis at pH 3.6 at right-angles to this. Ninhydrin-positive and phosphorus-containing substances were localized by appropriate sprays.

Recoveries of amino acids. Table 1 shows that satisfactory recoveries are obtained when a known mixture of amino acids in concentrations comparable with those found in the brain extracts were put through the analytical procedure just described.

Estimation of total nitrogen. This was carried out by a micro-Kjeldahl procedure (Chibnall, Rees & Williams, 1943).

Other analytical procedures

Estimation of total phosphorus. Total phosphorus in column effluents was determined by the method of Fiske & Subbarow (1925) after digestion with HClO₄. Samples of the fractions (2 ml.) were digested for 25 min. with 72% (w/v) HClO₄ (0.9 ml.), cooled and diluted with water (8.4 ml.); 5% (w/v) ammonium molybdate (0.5 ml.) and aminonaphtholsulphonic acid reagent (0.4 ml.) were added with shaking, and after 20 min. the extinction at 660 mμ was compared with that of suitable standards.

Estimation of lactate dehydrogenase. The enzyme was estimated spectrophotometrically on a Unicam SP.700 recording spectrophotometer as described by Johnson (1960) by following the fall in extinction at 340 mμ that accompanies the transfer of hydrogen from NADH₂ to pyruvate.

Estimation of potassium. This was carried out in a flame photometer (Baird Atomic model KY) on samples suitably diluted with water, with lithium as an internal standard.

Estimation of acetylcholine. This was carried out by using the dorsal muscle of the leech and the micro-assay procedure of Szerb (1961) as modified by Whittaker *et al.* (1964).

Fig. 1. Chromatograms of resin extract of homogenate from 0.28 g. of guinea-pig forebrain: (a) acidic and neutral amino acids; (b) basic amino acids. Continuous tracing, ninhydrin colour; histogram, total phosphorus. For details see the text. The arrow in (a) indicates the change from buffer at pH 3.25 and 30° to that at pH 4.25 and 50°. In (b), the portion of chromatogram shown was obtained at 30°; no further major components emerged when the temperature was raised to 50°. Identifications: 1-3 and 6, see the text; 4, taurine; 5, urea; 7, aspartate; 8, threonine; 9, serine; 10, asparagine + glutamine (not resolved); 11, glutamate; 12, glycine; 13, alanine; 14, γ -aminobutyrate; 15, NH_3 (derived from the resin extract).

RESULTS

Amino acids of brain homogenates and fractions

Identification of components. Fig. 1 shows a chromatogram obtained from an extract of a guinea-pig forebrain homogenate. Fifteen ninhydrin-positive peaks were obtained. With the exception of peaks 1-3 and 6 these were readily identified by means of runs with known substances (see the legend to Fig. 1). Peaks 1, 2 and 3 were approximately in the positions expected for phosphorylserine, glycylphosphorylethanolamine and phosphorylethanolamine respectively. Phosphorus analyses (upper tracing in Fig. 1a) showed that phosphorus was indeed present in the fractions corresponding to these peaks, but the molar P/NH_2 ratios varied across the peaks and were greater than unity. A fourth phosphorus-containing ninhydrin-negative component (peak 6a) appearing between peaks 6 and 7 was not further investigated.

Complex nature of material in peaks 1-3. The material in the fractions corresponding to peaks 1-3 and peak 6 was concentrated by adsorption on to and elution from resin as described in the Methods section and submitted to combined chromatography and electrophoresis on paper by the method of Dawson *et al.* (1963). The materials in peaks 1-3 were found to be complex mixtures. The phosphorus-containing material in peak 1 was mainly glycylphosphorylcholine (75%) and glycylphosphorylethanolamine (17%), and there were present in addition seven unidentified

non-phosphorus-containing ninhydrin-positive substances. The phosphorus compounds of peak 2 consisted almost entirely of glycylphosphorylcholine and glycylphosphorylethanolamine in approximately equal proportions, together with traces of phosphorylethanolamine and six other ninhydrin-positive substances all different from those in peak 1. Peak 3 consisted mainly of phosphorylcholine together with inorganic phosphate and five non-phosphorus-containing ninhydrin-positive substances. The ninhydrin-positive substances in these fractions are presumably acidic peptides; the heterogeneity of the fractions is not surprising in view of the poor resolving power of the column in this region.

Identification of peak 6. In contrast with peaks 1-3, the material in peak 6 appeared to consist of one main ninhydrin-positive compound. On keeping in aqueous solution it slowly underwent spontaneous hydrolysis with the release of glutamate; this suggested the presence of a γ -glutamyl residue. On refluxing for 24 hr. in a mixture containing hydrochloric acid (5N) and formic acid (10N) it was hydrolysed to glutamate with smaller amounts of aspartate, serine and glycine. This suggested that the main component might be γ -L-glutamyl-L-glutamate, recently isolated from ox brain by Kakimoto, Nakajima, Kanazawa, Takesada & Sano (1964) and appearing on their chromatograms in a similar position to our peak 6, 19 ml. after urea. A sample of synthetic glutamylglutamate kindly supplied by Dr Y. Kakimoto had the same retention

volume as peak 6 in our system. No indication was obtained for the presence of γ -glutamylglutamine, also isolated by Kakimoto *et al.* (1964).

Unidentified peaks labelled 'peptide' are seen in positions corresponding to our peak 6 in the chromatograms of hen-brain and bee-brain extracts published by Baker & Porcellati (1959) and Frontali (1962) respectively. Frontali's material yielded aspartate, serine, alanine, glycine and histidine as well as glutamate on hydrolysis.

Amino acids. In addition to the four peaks described above there were peaks corresponding to urea (no. 5), ammonia (derived from the resin eluate in the initial extraction procedure, no. 15) and nine amino acids, the most abundant of which (Table 2) was glutamate, followed, in decreasing concentration, by aspartate, γ -aminobutyrate, glutamine, glycine, serine, alanine, threonine and taurine. The glutamine peak may have contained some asparagine, since the system used could not resolve these two amides; however, the amount of asparagine in brain is known to be very low (Krebs, 1950). When larger amounts of material were chromatographed a number of other amino acids

could be detected as minor components. The concentrations found were similar to those reported by Tews *et al.* (1963) for dog brain except that those of taurine and amides were lower and those of γ -aminobutyrate, glycine, serine and alanine were somewhat higher.

Comparison with frozen brain. To determine the extent of any post-mortem changes in the concentration of free amino acids occurring during dissection and homogenization an analysis was made of extracts of brains of animals killed by immersion in liquid nitrogen. This method of extraction gives much higher values for ATP than homogenization in sucrose (Nyman & Whittaker, 1963) but not for other labile constituents such as acetylcholine (V. P. Whittaker & G. H. C. Dowe, unpublished work), which must therefore be protected more effectively before and during homogenization from enzymic destruction. A comparison of the results obtained with frozen brain and homogenates (Table 2) shows that the former contains about 40% more amino nitrogen, but the pattern of amino acid composition is similar in both preparations. The younger ages of the animals used in the experiments with frozen brain may account for part of the difference.

Table 2. *Amino acid analyses of frozen and homogenized guinea-pig forebrain*

The frozen brains were taken from young animals (wt. 200 g.); the sucrose homogenate was of brains from adult animals (wt. 450–600 g.).

Amino acid	Concn. (μ moles/g. of tissue)	
	Frozen brain	Sucrose homogenate
Non-P-containing amino compounds		
Glutamate	10.00	7.59
Glutamine	4.33	1.27
γ -Aminobutyrate	3.28	1.94
Aspartate	2.56	2.40
Glycine	0.90	1.00
Valine	0.62	Trace
Serine	0.46	0.88
Alanine	0.30	0.63
Threonine	0.29	0.23
Peak 6	0.23*	0.28
Isoleucine	0.08	Trace
Taurine	Trace	Trace
Urea	Trace	Trace
Total non-P-containing acids	23.10	16.22
P-containing peaks		
Peak 3	1.68*	0.99
Peak 1	0.42*	0.31
Peak 2	0.33*	0.15
Total	2.43*	1.45

* Leucine equivalent.

Distribution of amino acids in primary subcellular fractions

Table 3 shows the distribution in subcellular fractions of forebrain of the eight most abundant amino acids. Also included are the peptide in peak 6, the mixtures of ninhydrin-positive substances present in peaks 1–3, total nitrogen, lactate dehydrogenase and potassium.

Except for γ -aminobutyrate, the recoveries of the amino acids in the various fractions were close to 100%; γ -aminobutyrate showed recoveries of greater than 100% (124 and 126%) in the two experiments carried out, and glutamate was the only amino acid to show, on average, a recovery appreciably less than 100%. It seems possible that some decarboxylation of glutamate to γ -aminobutyrate may have occurred during the separation.

The distribution of the various amino acids in the different fractions is fairly uniform. Between 69% (serine) and 82% (threonine) are recovered in the high-speed supernatant fraction, S_3 . The remainder is distributed through the other fractions, the B fraction with two exceptions accounting for the next highest proportion. It is likely that the amino acid content of the particulate fractions may be accounted for by the presence in them of cytoplasm within unbroken cells (P_1), myelin-rich axonal fragments (P_1 and A) and synaptosomes (B). This is borne out by the similarity of their distribution pattern to that of lactate dehydrogenase

Table 3. *Distribution of amino acids, total nitrogen, lactate dehydrogenase and potassium in subcellular fractions of guinea-pig brain*

The results given are the means of two (non-phosphorus-containing amino compounds, total nitrogen), three (phosphorus-containing compounds, potassium) or four experiments (lactate dehydrogenase). For nomenclature of fractions see the text.

Compound	Concn. (μ moles/g. of tissue)	Recovery in fractions (% of homogenate)	Distribution in fractions (% of amount recovered)						Ratio of S_3 to total particulate
			P_1	A	B	C	P_3	S_3	
Non-P-containing amino compounds									
Glutamate	7.59	86	6	7	11	1	3	72	2.51
Aspartate	2.40	107	6	9	8	1	2	74	2.84
γ -Aminobutyrate	1.94	125	6	5	8	0	3	78	3.54
Glutamine	1.27	96	5	7	9	1	3	74	2.84
Glycine	1.00	98	6	9	10	2	2	71	2.45
Serine	0.88	93	5	9	12	2	3	69	2.22
Alanine	0.63	92	7	10	9	2	2	70	2.34
Peak 6	0.28*	97	5	4	11	1	2	77	3.35
Threonine	0.23	100	4	3	11	0	0	82	4.55
P-containing peaks									
Peak 3	0.99*	90	6	5	10	1	2	76	3.17
Peak 1	0.31*	116	17	6	14	4	4	55	1.22
Peak 2	0.15*	183	12	22	38	5	7	16	0.19
Other									
Total nitrogen	1.07†	100	29	11	18	7	8	26	0.35
Lactate dehydrogenase	47.6‡	90	10	6	9	2	3	70	2.34
Potassium	0.10†	105	9	6	10	1	2	73	2.70

* Leucine equivalent.

† mg.atoms/g.

‡ μ moles of NADH₂ disappearing/min./g. of tissue.

Table 4. *Relative concentrations of amino acids, lactate dehydrogenase and potassium in brain fractions*

For nomenclature of fractions see the text.

Fraction	Relative concn.		
	Amino acids	Lactate dehydrogenase	Potassium
P_1	0.20	0.34	0.31
A	0.67	0.54	0.54
B	0.55	0.50	0.56
C	0.15	0.29	0.14
P_3	0.27	0.37	0.40
S_3	2.83	2.69	2.81

(Table 3, l. 14) and potassium (Table 3, l. 15), both of which may be regarded as soluble cytoplasmic markers (Johnson & Whittaker, 1963; Ryall, 1964). Johnson & Whittaker (1963) found a somewhat sharper localization of particulate lactate dehydrogenase in fraction B than reported here; this is due to the fact that in their experiments fraction P_2 was washed with sucrose to remove the fraction of soluble cytoplasm, which was entrapped within myelin fragments. Unlike the synaptosome cytoplasm, this is readily washed out by sucrose.

Table 4 shows the concentrations of amino acids,

lactate dehydrogenase and potassium relative to the concentration of total nitrogen in the fractions. These values were obtained by dividing the distribution of the various components expressed as a percentage of the total recovered in the various fractions by the distribution of nitrogen expressed in similar units. The values for the amino acids were obtained from the means of the individual distribution values, a procedure justified by the closeness of these values to each other. The only fraction showing an enrichment of these components relative to the original homogenate (relative concentration, 1.00) is the soluble supernatant (S_3) fraction.

The subcellular distributions of the materials in peaks 1–3 have no great significance, since each peak comprises a complex mixture of substances. However, the material in peak 3 had a subcellular distribution closely similar to those of the non-phosphorus-containing amino compounds. The materials of peak 2 appeared to show a preferential localization in fraction B . However, the size of this peak in the chromatograms of the initial homogenate and the various fractions was low and variable and the 'recovery' was considerably in excess of 100%, suggesting autolytic breakdown. Further work will be necessary to determine whether there

is a component or components present in peak 2 that has a definite localization in fraction B.

Redistribution of amino acids during fractionation. Although there appeared to be no appreciable net synthesis or destruction of most of the amino acids during fractionation, as shown by recoveries at the end of the experiments close to 100%, the possibility was considered that redistribution of the amino acids could have taken place during fractionation either by leakage from particulate material into the supernatant or by adsorption from the supernatant on to particulate material. Comparisons were therefore made between the ratio of the total amounts recovered in the high-speed supernatant and the particulate fractions when complete fractionation was carried out and that obtained when the homogenate was centrifuged directly at 100 000g for 60 min. to give a high-speed supernatant (S) and a total particulate fraction (P). Table 5 shows that some leakage does take place during fractionation or even during standing at 0°: the S/P ratio increased from 1.94 when the homogenate was submitted to high-speed centrifuging immediately after preparation to 2.45 after standing for 2–6 hr. as compared with a ratio 2.70 as a result of full fractionation. These increases are smaller than those observed with hydroxytryptamine (Michaelson & Whittaker, 1963).

Effect of homogenizing in saline. Elliott & van Gelder (1960) reported that 'factor I' (γ -aminobutyrate) remained 70% bound in saline homogenates but only 30% bound in sucrose. We

therefore determined one series of S/P ratios by using a homogenate made in 0.9% sodium chloride. The results are presented in Table 5. Except for the amides, the amino acid content was somewhat lower than in the experiments presented in Table 2, but the general pattern of distribution was similar; 63% or more of the amino acids was recovered in the high-speed supernatant. The proportion in the supernatant was, however, usually somewhat lower in saline than in sucrose.

*Distribution of amino acids in subfractions
from disrupted synaptosomes*

The results so far presented show that the free amino acids of brain tissue have a subcellular distribution similar to that of soluble cytoplasmic markers such as lactate dehydrogenase and potassium. Their presence in the synaptosome and other particulate fractions could be accounted for by the presence in these fractions of entrapped cytoplasm. It was therefore decided to study the distribution of the amino acids in density-gradient fractions of disrupted synaptosomes, to see whether the amino acids again had a distribution similar to the soluble cytoplasmic markers. Particular attention was paid to the synaptic-vesicle fraction because of the presence of bound acetylcholine in this fraction and the possible role of the synaptic vesicles in the binding of transmitter substances generally.

Two series of experiments were carried out, with synaptosomes prepared from whole brain and

Table 5. *Distribution of amino acids between particulate and high-speed supernatant fractions*

The supernatant/particulate ratio for the first entry is the ratio $S_3/(P_1 + A + B + C + P_3)$ calculated from the results given in Table 2, and for the remaining entries it is S/P. For nomenclature of fractions see the text.

Amino acids	Time from homogenizing to final sampling (hr.)	Concn. in homogenate (μ moles or μ g.atoms)	Amino acids recovered in fractions (% of homogenate)	Supernatant/ particulate concentration ratio
Full fractionation				
Total non-P-containing amino acids	5	15.94	96	2.70
High-speed centrifuging only				
Total carboxylic amino N	1	16.22	111	1.94
	3		115	2.45
	7		102	2.45
High-speed centrifuging only, saline homogenate				
Non-P-containing amino acids:				
Glutamate	1	5.66	104	2.50
Amides		3.52	69	2.51
Aspartate		2.23	88	2.33
γ -Aminobutyrate		1.47	104	1.78
Serine		0.74	83	2.07
Glycine		0.69	100	1.96
Alanine		0.55	116	2.86
Threonine		0.09	120	2.09
Total non-P-containing amino acids		14.95	94	2.33

Table 6. *Distribution of amino nitrogen and marker substances in subfractions of disrupted synaptosomes*

For nomenclature of fractions see the text.

Component	No. of experi- ments	Units	Parent fraction	Amount recovered		Distribution (% of recovered amount)									
				(units/g. of original tissue)	(% of parent fraction)										
				<i>O</i>	<i>D</i>	<i>E</i>	<i>F</i>	<i>G</i>	<i>H</i>	<i>I</i>	<i>J</i>	<i>D_s</i>	<i>D_p</i>		
Whole brain															
Amino N	1	μg.atoms	<i>W_s</i>	3.34	102	55	17	7	6	6	7	2	—	—	—
Lactate dehydro- genase*	3	μmoles of NADH ₂ dis- appearing/min.	<i>W_s</i>	5.32	71	74	16	3	1	0	0	3	—	—	—
Potassium*	1	μg.atoms	<i>W_s</i>	6.50	100	75	14	4	5	2	0	0	—	—	—
Acetylcholine*	4	mμmoles	<i>W_s</i>	1.66	81	2	30	8	5	11	35	9	—	—	—
Total nitrogen*	11	mg.atoms	<i>W_s</i>	0.17	84	28	12	8	12	12	15	13	—	—	—
Cortex															
Amino N	2	μg.atoms	<i>P₂</i>	4.00	84	52	21	—	—	—	—	—	27	—	—
	2	μg.atoms	<i>D</i>	0.72	89	—	—	—	—	—	—	—	—	73	27
Potassium	1	μg.atoms	<i>P₂</i>	8.67	87	55	19	—	—	—	—	—	26	—	—
Acetylcholine	1	mμmoles	<i>P₂</i>	5.56	84	8	49	—	—	—	—	—	43	—	—
Total N	3	mg.atoms	<i>P₂</i>	0.18	82	20	10	—	—	—	—	—	70	—	—

* Results of Whittaker *et al.* (1964) included for comparison.

cortex respectively. The results are summarized in Table 6. Most of the amino nitrogen was recovered in each series in fraction *O* containing the soluble cytoplasmic component of the synaptosomes. In the whole-brain series, the distribution is similar to that of lactate dehydrogenase and potassium, except that relatively more is present in the fractions containing membrane fragments (*E-G*) and incompletely disrupted synaptosomes (*H*). The amount of all these substances present in fraction *D* can be accounted for by diffusion from fraction *O*. The distribution contrasts with that of bound acetylcholine (1. 4), which shows a bimodal distribution with peaks in the fractions containing synaptic vesicles (*D*) and incompletely disrupted synaptosomes (*H*). A comparison with the total nitrogen distribution shows that the maximum concentration of amino acids with respect to other nitrogenous constituents of the fractions was also in fraction *O*.

In the series with disrupted cortical synaptosomes, a similar trend was evident, the distribution in the gradient being similar to potassium and the richest fraction being the soluble cytoplasmic fraction *O*. The contrast with bound acetylcholine was even more marked, almost half of the latter being recovered in the synaptic-vesicle fraction.

However, the possibility still remained that the synaptic vesicles might have concentrated particular amino acids from the mixture of free amino acids present in the synaptosome fraction whose presence there would have been masked by the larger amount

Table 7. *Composition of free amino acid pool in subfractions of synaptosomes from guinea-pig cortex*

For nomenclature of fractions see the text.

	Percentage composition of amino acid pool		
	P ₂	O	D _s
Glutamate	37	35	31
Amides	24	13	10
Aspartate	15	5	10
γ-Aminobutyrate	7	20	5
Serine	6	13	11
Glycine	5	10	21
Alanine	4	4	7
Threonine	2	<1	5
Total amino N (μmoles/g.)	3.29	1.23	0.28
As % of total amino compounds present in fraction	71	59	53

of free amino acids recovered in fraction *O*. Consequently in five experiments the *D* fraction from brain cortex was diluted with an equal volume of water and centrifuged at 100000g for 60 min. to sediment the synaptic vesicles. The amino acid distribution was then determined in the precipitate and supernatant (fractions *D_p* and *D_s*). In previous work with whole brain (Whittaker *et al.* 1964) 80% of the recovered acetylcholine had sedimented into fraction *D_p*. In three out of five of these experiments an interfering substance was released into fraction

D_4 during this procedure that did not appear as an amino acid peak on subsequent chromatography. Consequently the total amino nitrogen of this fraction could not be determined by the ninhydrin method and was instead obtained by summing the amino acid peaks. The distribution studies (Table 6, l. 7, columns 15 and 16) indicated that only 27% of the total amino acid originally in the synaptic-vesicle fraction sedimented with the synaptic vesicles. This represents less than 0.5% of the amino acid nitrogen of the original homogenate.

Amino acid analyses of cortical fractions P_2 , O and D_8 (Table 7) showed that in all three fractions the same eight amino acids accounted for 53–71% of the total amino compounds with glutamate as the most abundant. The low concentration of ninhydrin-positive material in fraction D_p did not permit an analysis of this fraction.

DISCUSSION

Seven amino acids together account for over 80% of the free amino compounds of sucrose homogenates of guinea-pig brain. These are, in decreasing order of abundance: glutamate, aspartate, γ -aminobutyrate, glycine, serine, alanine and threonine. In addition, there are appreciable quantities of amide (presumably glutamine).

Of these compounds, glutamate and aspartate can stimulate nerve cells to discharge electrically when applied iontophoretically by means of microelectrodes, and γ -aminobutyrate, alanine and glycine, again in decreasing order of effectiveness, depress central neurones when similarly applied (Curtis & Watkins, 1960; Krnjević, 1965). The uniquely high concentrations of glutamate and γ -aminobutyrate, respectively the most effective excitant and depressant compounds of this class found in brain tissue, have led to speculations that these compounds may be the chemical transmitter substances at the many excitatory and inhibitory endings at which no other transmitter has so far been implicated.

The subcellular fractionation studies do not, however, demonstrate, as they did for acetylcholine, a selective localization of these, or, indeed, of any of the other amino acids in the fraction in which the isolated nerve endings are concentrated; 69–82% was recovered in the high-speed supernatant fraction and the distribution in the other fractions closely parallels that of the soluble cytoplasmic markers lactate dehydrogenase and potassium. This finding is in agreement with the results of Ryall (1964) for glutamate and γ -aminobutyrate in rat brain and of Weinstein *et al.* (1963) for γ -aminobutyrate in mouse brain. A reasonable inference is that the amino acid content of the particulate fractions merely reflects the occlusion

of cytoplasm within organized cell fragments (mainly nerve endings) in these fractions.

The results of hypo-osmotic disruption of synaptosomes (Table 6) confirm these conclusions; over half (52–55%) of the amino nitrogen of the preparations was recovered in the fraction (O) corresponding to soluble cytoplasm, compared with 55–75% of the potassium. The next richest fraction was that containing the synaptic vesicles (D), which have been shown to contain acetylcholine; but when the vesicles were sedimented along with their contained acetylcholine most (73%) of the amino compounds remained in the supernatant. No evidence was obtained for a selective localization of any particular amino acid in any of these sub-fractions; pharmacologically active and inactive amino acids were distributed in an essentially similar way.

These findings, together with the fact that no amino acid so far tested has duplicated exactly the post-synaptic electrical effects of synaptic transmission (Curtis, 1962, 1963), have been regarded as evidence against a specific transmitter role for these substances in the mammalian central nervous system (Crawford & Curtis, 1964). Nevertheless, a note of caution should be sounded with regard to the interpretation of subcellular distribution studies. Although a relatively small proportion of the total tissue glutamate and γ -aminobutyrate is recovered in the synaptosome and synaptic-vesicle fractions, Krnjević & Whittaker (1965) directly demonstrated by means of electroendosmosis from micropipettes that there is sufficient of both these substances in extracts of these fractions to produce effects on single cortical neurones of the guinea pig. From synaptosome (F. Clementi, M. N. Sheridan & V. P. Whittaker, unpublished work) and synaptic-vesicle (Whittaker & Sheridan, 1965) counts they were able to estimate that excitant effects were being obtained with rates of glutamate release corresponding to the content of about 700 synaptosomes/sec. and depressant effects with rates of γ -aminobutyrate release corresponding to that of about 350 synaptosomes/sec. Synaptic activation at these rates was considered to be well within the possible physiological limit.

There is, in fact, no reason to assume that the mechanisms of storage and release of all central transmitters must be the same as those of acetylcholine. The storage mechanism might, for example, involve continuous active uptake; under the conditions of subcellular fractionation in an essentially ion-free medium at 0°, outward diffusion from storage sites might occur, leading to a distribution of transmitter indistinguishable from that of a soluble cytoplasmic constituent. Sano & Roberts (1963) have obtained evidence for a Na^+ -dependent uptake of labelled γ -aminobutyrate in

mouse-brain particles; a more detailed analysis (Varon, Weinstein & Roberts, 1964) suggested that endogenous γ -aminobutyrate is concomitantly released into the incubation medium. It is thus noteworthy that the γ -aminobutyrate-forming enzyme glutamate decarboxylase, which, as a high-molecular-weight substance, may be less liable to redistribution during fractionation, has been reported to be localized in a dense synaptosome fraction (Weinstein *et al.* 1963; Salganicoff & De Robertis, 1963; van den Berg, 1964).

Any transmitter that is not highly concentrated at nerve endings will show a distribution approximating to that of a soluble cytoplasmic marker if the neurones containing the transmitter are represented in the sample as a whole and not simply by their nerve endings (for discussion see Lavery, Michaelson, Sharman & Whittaker, 1963; Krnjević & Whittaker, 1965). Inhibitory endings, particularly, are likely to originate according to recent views (Eccles, 1964) from cells (e.g. basket or stellate cells) with short axons making axosomatic contacts with neighbouring cells; thus the inhibitory neurones as a whole will be included in the preparation. It is also not clear to what extent axosomatic as contrasted with axodendritic endings are pinched off intact during homogenization. In view of all these considerations it is unlikely that the evidence from subcellular distribution is critical enough at the present time to provide an adequate basis for rejecting glutamate and γ -aminobutyrate as transmitters.

In the course of the present work, the presence in brain homogenates of appreciable amounts of a peptide that undergoes spontaneous hydrolysis to glutamate and of at least 18 unidentified ninhydrin-positive compounds was noted. Apart from the peptide the subcellular distribution of none of these substances has been followed up, nor is it known if any of them have effects on central neurones.

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