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THE REQUIREMENTS OF THE BRAIN FOR SOME AMINO ACIDS

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SUMMARY

1. A constant specific activity of radioactively labelled amino acids was maintained in the circulation by means of a new technique devised for this purpose. This has made it possible to measure accurately the entry rates of amino acids into the brain *in vivo*.

2. The rates of entry into the brain of seven nutritionally non-essential amino acids were measured.

3. Glycine and proline enter the brain relatively slowly, at rates comparable to those of amino acids which are not normally found in the blood. Thus their entry is due mainly if not entirely to passive diffusion.

4. Serine (which is used by the brain to make glycine) and alanine (which is used to make glutamate and aspartate) enter the brain as rapidly as the essential amino acids and thus, although not essential for the body as a whole, appear to be essential for the brain.

5. It is suggested that those amino acids that the brain is able to synthesize have low rates of entry, even though they are present at high concentrations in the plasma, but that the transport systems for those amino acids that are not synthesized in the brain ensure rapid entry at rates that are related to the rates of cerebral utilization.

INTRODUCTION

Much work has been done on the cerebral metabolism of amino acids *in vitro* (Lajtha, 1970) but it is difficult to assess the significance of many of the findings from these studies without accurate information about the rates at which these substances are transported into the brain *in vivo*. For example, Shank & Aprison (1970) have demonstrated the existence of a metabolic pathway in the brain for the interconversion of glycine and serine but this leaves open the question as to which way this pathway

operates in life, and also whether the pathway is a major one by which important cerebral metabolic needs are met. Measurements of rates of transport *in vitro* (Neame, 1968; Cohen & Lajtha, 1972) are not able to answer this sort of question, while damage to the tissue, which is inevitable with *in vitro* preparations, is likely to exaggerate the possible rôle of diffusion. Such damage may well explain the relatively rapid entry of 2-aminoisobutyrate (which is not found in the blood) into brain slices (Blasberg, 1968) compared with its slow entry into the brain *in vivo* (Baños, Daniel, Moorhouse & Pratt, 1973). The effect of damage to the cells is illustrated in the case of the olfactory bulb preparation where the uptake of amino acids is greatly increased if the bulb is sliced (Neidle, Kandera & Lajtha, 1973).

We have shown earlier (Baños, Daniel, Moorhouse & Pratt, 1970, 1973), as has Oldendorf (1971), that the entry rate of essential amino acids into the living brain is high presumably in order to meet the needs of the brain for protein synthesis (the quantities of amino acids involved in neurotransmitter metabolism are minute (Carlsson, 1974) compared with those used in protein metabolism). In the present paper we have studied especially those amino acids which are not essential for the nutrition of the body so that we might find out whether their rates of entry throw light on the activity, in the living animal, of the relevant cerebral pathways of amino acid metabolism.

METHODS

For all experiments Wistar rats aged between 6 and 9 weeks were used. They were given food (Oxoid breeding pelleted diet containing 20·4% of protein) and water *ad lib.* Amino acids (obtained from Koch-Light Laboratories Ltd, Bucks., England) and all other chemicals used were of analytical grade. The amino acids shown in Table 1 and labelled with [^{14}C] or [^{35}S] were obtained from the Radiochemical Centre, Amersham, England.

The animals were anaesthetized with ether or with sodium pentobarbitone (35 mg/kg). Blood samples (usually 0·2 ml.) were withdrawn through a cannula which was inserted into a femoral artery and one or both femoral veins were cannulated so that injections of radioactively labelled and unlabelled amino acids could be given. A steady level of amino acid was maintained in the circulation by means of a method described by Daniel, Donaldson & Pratt (1974, 1975) in which the substance was injected intravenously by means of a syringe with a variable drive (Pratt, 1974). At the end of the experiment the jugular veins were opened and most of the blood was rapidly washed out of the brain with warm saline (for 20 sec) by means of a pressure apparatus connected to the arterial cannula (Daniel, Love, Moorhouse, Pratt & Wilson, 1974). The animal was killed by decapitation and the brain rapidly removed, washed briefly in saline, blotted and frozen in hexane at $-70°$ C. Samples were stored at $-15°$ C until they could be assayed. The radioactivity in brain and blood plasma was counted.

Analysis of tissues. Two methods were used to prepare the tissues and plasma for radioactive assay: 2 ml. of a solution of an organic base (Soluene X 100; Packard

Instruments) were added to a weighed sample of brain tissue in a glass scintillation counting vial. When the tissue had dissolved, glacial acetic acid (0·1–0·2 ml.) was added to neutralize the organic base and 15 ml of a scintillation mixture containing 5 g 2,5-diphenyloxazole and 0·3 g 1,4-bis(2(4-methyl-5-phenyloxazolyl)) benzene per litre of toluene were added. Plasma was rapidly separated from heparinized blood, 0·1 or 0·05 ml. aliquots were added to 1 ml. Soluene X 100 in glass scintillation counting vials and prepared in a similar manner for assay of the radioactivity. Alternatively, samples of brain or plasma were oxidized with $HClO_4$ and H_2O_2 and the liquid scintillation reagents were made up to a total volume of 16 ml. with ethylene glycol monoethyl ether as described by Mahin & Lofberg (1966). The radioactivity in the samples was measured in an automatic scintillation spectrophotometer (Tricarb model 3375, Packard Instruments). No difference was found between the results obtained by these two methods. Where necessary a small correction was made for dry quenching of the scintillation, using the channel ratio method for this purpose.

To prepare samples for measurement of the free amino acid, freshly separated blood plasma was added to 5 vol. 1% picric acid solution and brain samples were homogenized in the same solution. The picric acid was removed by adsorption on Dowex 50 resin and the amount of each amino acid in the extract was measured, after separation on an ion-exchange column, by means of an automatic amino acid analyser (BC 200, LKB), lithium citrate buffers (Atkin & Ferdinand, 1970) being used for the elution.

Crude protein was extracted from the cerebral hemispheres of two rats. Each cerebral hemisphere was ground up with 20 times its weight of chloroform-methanol (2:1 v/v). The suspension was filtered and the residual protein washed with chloroform-methanol and finally with 70% (v/v) of aqueous methanol. After the residual solvent had evaporated the crude cerebral protein was dried to a constant weight in a desiccator. Weighed samples (about 1·0 mg) were hydrolysed with 6N-HCl by heating *in vacuo* at 110° C for periods varying from 6 to 48 hr. Each hydrolysate was evaporated to dryness to remove the HCl, made up to 2 ml. with lithium citrate buffer (pH 2·15) and analysed for amino acid content. Corrections were made for losses of amino acid during hydrolysis.

RESULTS

A constant level of [^{14}C]glycine was maintained in the bloodstream in a series of experiments that varied from 1 to 10 min in duration. The ratio of the radioactivity that accumulated in the brain, R_c, to the mean level of radioactivity in the plasma, R_p, was determined and it was found that over the first few minutes the ratio R_c/R_p increased in proportion to the duration of the injection. As the injection was prolonged the radioactivity accumulated more slowly in the brain (Fig. 1). Similar results were obtained with the other amino acids (e.g. Fig. 2).

The slowing down of the rate of accumulation of radioactivity in the longer experiments was assumed to be due to the return to the circulation of some of the radioactivity that had entered the brain. Thus it was felt that only the initial, almost linear, parts of the curves provided an accurate measure of the entry rate into the brain, and in subsequent experiments the duration of the injection was limited to 1 or 3 min. Such

short experiments also have the advantage of reducing the likelihood of any error due to the metabolism of the labelled amino acid before it enters the brain.

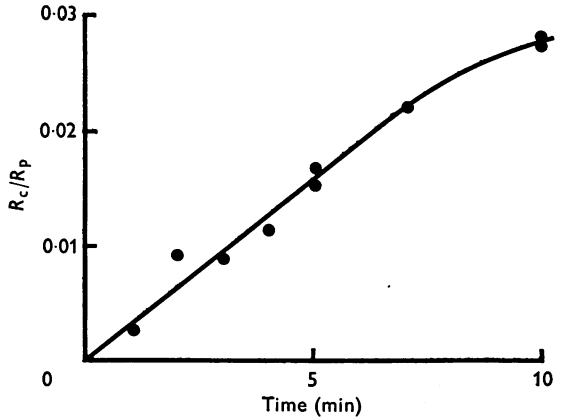

Fig. 1. The effect of increasing the length of time of injection on the entry of [^{14}C]glycine into the brain. Each point represents a separate experiment.

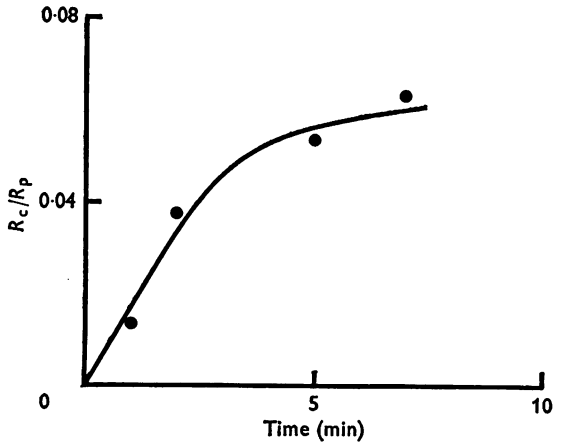

Fig. 2. The effect of increasing the length of time of injection on the entry of [^{14}C]L-cysteine into the brain. Each point represents a separate experiment.

Measurement of entry rates

The entry rate, v, of an amino acid into the brain was calculated as follows:

$$v = \frac{sR_c}{R_p t},$$

TABLE 1. Data on the non-essential amino acids used and the range of concentrations in the blood plasma over which entry rates were measured

Amino acid	Concentration in normal plasma ± S.E. (number of determinations in parentheses) (n-mole ml.$^{-1}$)	Molecular weight	Range of concentration in plasma over which entry rate was measured (n-mole ml.$^{-1}$)	Radioactive label (with position in the molecule in parentheses)
L-Serine	186 ± 14 (11)	105	170–4000	^{14}C (U)
L-Cysteine	105 ± 15 (13)	121	100–200	^{14}C (U) or ^{35}S
L-Citrulline	61 ± 10 (3)	175	60–200	^{14}C (carbamyl)
L-Alanine	310 ± 41 (14)	89	300–500	^{14}C (U)
L-Glutamate	156 ± 23 (8)	147	140–200	^{14}C (U)
Glycine	224 ± 17 (14)	75	200–400	^{14}C (U)
L-Proline	162 ± 10 (10)	115	160–800	^{14}C (U)

TABLE 2. Comparison of standardized entry rates at normal plasma concentrations for the amino acids studied together with the concentration of each amino acid free in the cerebral cells and combined in cerebral protein

Amino acid	Standardized entry rate (n-mole min^{-1} g^{-1} brain)	Entry rate at mean normal plasma concentration (n-mole min^{-1} g^{-1} brain)	Nutritional category	Mean content of amino acid in brain (n-mole g^{-1} brain)	Mean content of amino acid in crude brain protein (n-mole mg^{-1} protein)
[1]L-Serine	1·28 ± 0·28 (8)	2·38	Non-essential	1040	573
[2]L-Cysteine	1·11 ± 0·2 (5)	1·16	Non-essential	38	—
[3]L-Citrulline	0·93 ± 0·3 (3)	0·57	Non-essential	36	—
[4]L-Alanine	0·87 ± 0·2 (6)	2·70	Non-essential	602	756
[4]L-Glutamate	0·63 ± 0·26 (4)	0·98	Non-essential	8631	1168
[1]Glycine	0·29 ± 0·01 (14)	0·65	Non-essential	697	684
[5]L-Proline	0·19 ± 0·02 (7)	0·31	Non-essential	74	450

1. Brain can readily convert serine into glycine or vice versa (Shank & Aprison, 1970).

2. Brain can synthesize cysteine from methionine (Gaitonde & Richter, 1956).

3. Although enzymes for the conversion of arginine to citrulline are present in brain, the activity is low when compared with the liver (Levin, 1971, p. 68).

4. Brain contains transaminases catalysing the conversion of alanine, aspartate and glutamate (Balázs, 1965).

5. The brain is thought to be able to make proline from glucose or from other amino acids (Strecker, 1970).

where s is the concentration of the amino acid in the plasma and t is the duration of the injection of radioactively labelled amino acid.

In these experiments the radioactive labelling was of high specific activity so that the concentration in the plasma of the amino acid under investigation was not raised appreciably above the normal endogenous level.

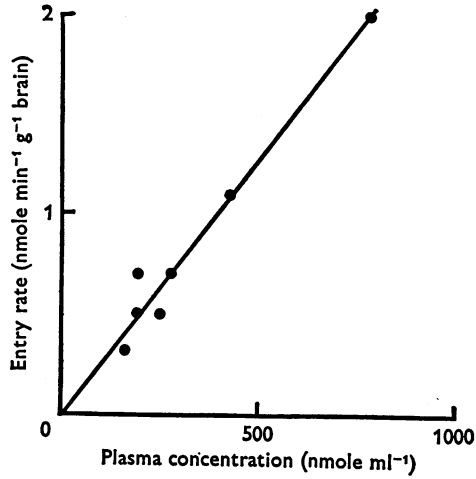

Fig. 3. The effect of increasing the concentration of L-proline in the plasma on the rate of its entry into the brain. Each point represents a separate experiment.

The effect of the concentration of the amino acid in the plasma on its rate of entry into the brain

Fig. 3 shows that the rate of entry of L-proline into the brain increased approximately in proportion to its concentration in the plasma, at least up to a plasma concentration of about 1 μmole ml.$^{-1}$ A similar relationship between plasma concentration and rate of entry into the brain was found for all the other amino acids in Table 1; i.e. evidence of saturation of the carrier mechanism could not be detected at these plasma concentrations.

The rates of entry into the brain of the various amino acids

The normal concentrations of the amino acids in the plasma are shown in Table 1. Since the entry rate of each amino acid was approximately proportional to its concentration in the plasma (over the range shown in Table 1) the convention of standardized entry rates was used in order to permit direct comparisons to be made between various amino acids (see Baños *et al.* 1973). The standardized entry rate was calculated from eqn. (1), as if the concentration of the amino acid in the plasma was 100 n-mole ml.$^{-1}$ rather than the actual value. This arbitrary reference

level is close to the mean normal plasma concentrations of many of the amino acids (Table 1). From these standardized rates (Table 2, column 2) the entry rates at the normal plasma concentrations were calculated (Table 2, column 3). The mean concentration of each amino acid free in brain and the proportions of each of the amino acids present in combined form in a sample of mixed proteins separated from the cerebral hemispheres are also shown in Table 2.

DISCUSSION

The findings reported here (Table 2) on the entry of seven non-essential amino acids into the bran of the living rat extend our previous work (Baños *et al.* 1973). As to how amino acids enter the brain in the living animal, there is now evidence that an amino acid can saturate its own transport mechanism (Baños *et al.* 1970) and that one amino acid can saturate the transport mechanism of a chemically related amino acid (Baños, Daniel, Moorhouse & Pratt, 1974; Baños, Daniel & Pratt, 1974; Baños, Daniel, Moorhouse, Pratt & Wilson, 1974). This supports the theory that carrier-mediated transport systems (Neame & Richards, 1972) convey many of these substances into the brain. Such transport systems may well include facilitated exchange mechanisms of the kind described by Christensen, Cespedes, Handlogten & Ronquist (1973). However, it must not be forgotten that diffusion may also play a small part in the entry of amino acids into the cerebral tissues. Clearly, free diffusion does not occur, but what seems probable is that amino acids diffuse slowly into the brain during life through a barrier that is not completely permeable.

Since all the amino acids listed in Table 2, and the fifteen other amino acids that were studied previously (Baños *et al.* 1973), are essentially similar in their physico-chemical properties, we assume that they are likely to diffuse into the brain at approximately similar rates. Wilson (1974) estimated that in the case of five amino acids (L-serine, L-isoleucine, L-leucine, L-threonine and L-valine) the component of the total entry rate that is due to diffusion is between 0·05 and 0·2 n-mole min^{-1} g^{-1} brain for every 100 n-mol $ml.^{-1}$ of amino acid in the plasma. As the entry rates of these five amino acids are well above this range a considerable proportion of the entry must be due to carrier-mediated transport (see Table 2 of this paper and Table 1 in Baños *et al.* 1973). However, the standardized entry rates of several amino acids fall within this range, or close to it, e.g. glycine and L-proline (Table 2). Other amino acids whose standardized rates of entry are within this low range are taurine (an amino acid not found in protein) and two amino acids that are not normally found in the

body, 2-aminoisobutyrate and DL-2-aminoadipate (Baños *et al.* 1973). It seems likely, therefore, that under normal conditions these few amino acids enter the brain mainly by diffusion and that carrier-mediated transport systems play little part in their entry.

A comparison of the low entry rates of this group with the higher entry rates of the other five amino acids in Table 2 and of the remaining twelve amino acids in the series previously studied (Table 1 in Baños *et al.* 1973) suggests that all those amino acids whose entry rates are higher are transported into the brain by carrier-mediated systems and that diffusion plays only a small part in their entry. We have shown previously that the ten essential and semi-essential amino acids (Rose & Cox, 1924; Borman, Wood, Black, Anderson, Oesterling, Womack & Rose, 1946) which cannot be synthesized by cerebral cells must enter the brain from the circulating blood (Baños *et al.* 1973). The present findings show that among the non-essential amino acids not only tyrosine, which cannot be synthesized by the brain (Gal, Armstrong & Ginsberg, 1966; Guroff & Abramowitz, 1967), but also serine and alanine (Table 2) enter the brain at rates comparable to those of the essential amino acids. This indicates that for the cerebral cells these three amino acids should be regarded as essential even though they are not essential for the body as a whole. The brain can convert serine into glycine and vice versa (Shank & Aprison, 1970) but the high entry rate of the former (Table 2) suggests that in life the cerebral cells synthesize glycine from serine and not the reverse. There may be a comparable explanation for the differences in the rates of entry of alanine, aspartate and glutamate. The brain can interconvert these three amino acids (Balázs, 1965), but since the rate of entry of alanine is so much higher than the entry rates of the other two (Table 2, column 3 and Baños *et al.* 1973) it seems likely that the cerebral tissues make aspartate and glutamate from alanine.

We conclude that those amino acids that the brain can synthesize have low rates of entry, even though they are present at high concentrations in the plasma (e.g. proline and glycine). On the other hand the transport systems for those amino acids that the cerebral cells either cannot or do not synthesize but which they use in considerable quantities are geared to ensure rapid entry at rates that are related to the rates of cerebral utilization.

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REFERENCES

ATKIN, G. E. & FERDINAND, W. (1970). Accelerated amino acid analysis: studies on the use of lithium citrate buffers and the effect of *n*-propanol, in the analysis of physiological fluids and protein hydrolyzates. *Analyt. Biochem.* **38**, 313–329.

BALÁZS, R. (1965). Control of glutamate metabolism. The effect of pyruvate. *J. Neurochem.* **12**, 63–76.

BAÑOS, G., DANIEL, P. M., MOORHOUSE, S. R. & PRATT, O. E. (1970). The passage of amino acids into the rat's brain. *J. Physiol.* **210**, 149*P*.

BAÑOS, G., DANIEL, P. M., MOORHOUSE, S. R. & PRATT, O. E. (1973). The influx of amino acids into the brain of the rat *in vivo*: the essential compared with some non-essential amino acids. *Proc. R. Soc.* B **183**, 59–70.

BAÑOS, G., DANIEL, P. M., MOORHOUSE, S. R. & PRATT, O. E. (1974). Inhibition of entry of some amino acids into the brain, with observations on mental retardation in the aminoacidurias. *Psychol. Med.* **4**, 262–269.

BAÑOS, G., DANIEL, P. M., MOORHOUSE, S. R., PRATT, O. E. & WILSON, P. (1974). Inhibition of neutral amino acid entry into the brain of the rat *in vivo*. *J. Physiol.* **237**, 22–23*P*.

BAÑOS, G., DANIEL, P. M. & PRATT, O. E. (1974). Saturation of a shared mechanism which transports L-arginine and L-lysine into the brain of the living rat. *J. Physiol.* **236**, 29–41.

BLASBERG, R. G. (1968). Specificity of cerebral amino acid transport: a kinetic analysis. In *Progress in Brain Research*, ed. LAJTHA, A. & FORD, D. H., **29**, pp. 245–256. Amsterdam, London, New York: Elsevier.

BORMAN, A., WOOD, T. R., BLACK, H. C., ANDERSON, E. G., OESTERLING, M. J., WOMACK, M. & ROSE, W. C. (1946). The rôle of arginine in growth with some observations on the effects of argininic acid. *J. biol. Chem.* **166**, 585–594.

CARLSSON, A. (1974). The *in vivo* estimation of rates of tryptophan and tyrosine hydroxylation: effects of alterations in enzyme environment and neuronal activity. In *Aromatic Amino Acids in the Brain*, ed. WOLSTENHOLME, G. E. W. & FITZSIMONS, D. W., p. 117. Amsterdam: Elsevier.

CHRISTENSEN, H. N., DE CESPEDES, C., HANDLOGTEN, M. E. & RONQUIST, G. (1973). Energization of amino acid transport, studied for the Ehrlich ascites tumour cell. *Biochem. biophys. Acta* **300**, 487–522.

COHEN, S. R. & LAJTHA, A. (1972). Amino acid transport. In *Handbook of Neurochemistry*, vol. 7, ed. LAJTHA, A., ch. 21, pp. 543–572. Pathological chemistry of the nervous system. New York, London: Plenum Press.

DANIEL, P. M., DONALDSON, J. & PRATT, O. E. (1974). The rapid achievement and maintenance of a steady level of an injected substance in the blood plasma. *J. Physiol.* **237**, 8–9*P*.

DANIEL, P. M., DONALDSON, J. & PRATT, O. E. (1975). A method for injecting substances into the circulation to reach rapidly and to maintain a steady level. *Med. & biol. Engng* (in the Press).

DANIEL, P. M., LOVE, E. R., MOORHOUSE, S. R., PRATT, O. E. & WILSON, P. (1974). A method for rapidly washing the blood out of an organ or tissue of the anaesthetized living animal. *J. Physiol.* **237**, 11–12*P*.

GAITONDE, M. K. & RICHTER, D. (1956). The metabolic activity of the proteins of the brain. *Proc. R. Soc.* B **145**, 83–99.

GAL, E. M., ARMSTRONG, J. C. & GINSBERG, B. (1966). The nature of *in vitro* hydroxylation of L-tryptophan by brain tissue. *J. Neurochem.* **13**, 643–654.

GUROFF, G. & ABRAMOWITZ, A. (1967). A simple radioisotope assay for phenylalanine hydroxylase. *Analyt. Biochem.* **19**, 548–555.

LAJTHA, A. (1970). Metabolic reactions in the nervous system. In *Handbook of Neurochemistry*, vol. 3, ed. LAJTHA, A., ch. 6–11. New York, London: Plenum Press.

LEVIN, B. (1971). Hereditary metabolic disorders of the urea cycle. In *Advances in Clinical Chemistry*, vol. 14, ed. BODANSKY, O. & LATNER, A. L., pp. 65–143. New York, London: Academic Press.

MAHIN, D. T. & LOFBERG, R. T. (1966). A simplified method of sample preparation for determination of tritium, carbon-14 or sulfur-35 in blood or tissue by liquid scintillation counting. *Analyt. Biochem.* **16**, 500–509.

NEAME, K. D. (1968). Transport, metabolism and pharmacology of amino acids in brain. In *Applied Neurochemistry*, ed. DAVISON, A. N. & DOBBING, J., pp. 119–177. Oxford: Blackwell Scientific Publications.

NEAME, K. D. & RICHARDS, T. G. (1972). *Elementary Kinetics of Membrane Carrier Transport.* Oxford: Blackwell Scientific Publications.

NEIDLE, A., KANDERA, J. & LAJTHA, A. (1973). The uptake of amino acids by the intact olfactory bulb of the mouse. A comparison with tissue slice preparations. *J. Neurochem.* **20**, 1181–1193.

OLDENDORF, W. H. (1971). Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection. *Am. J. Physiol.* **221**, 1629–1639.

PRATT, O. E. (1974). An electronically controlled syringe drive for giving an injection at a variable rate according to a preset programme. *J. Physiol.* **237**, 5–6*P*.

ROSE, W. C. & COX, G. J. (1924). The relation of arginine and histidine to growth. *J. biol. Chem.* **61**, 747–773.

SHANK, R. P. & APRISON, M. H. (1970). The metabolism *in vivo* of glycine and serine in eight areas of the rat central nervous system. *J. Neurochem.* **17**, 1461-1475.

STRECKER, H. J. (1970). Biochemistry of selected amino acids. In *Handbook of Neurochemistry*, vol. 3, ed. LAJTHA, A. Metabolic reactions in the nervous system, ch. 6, pp. 173–207. New York, London: Plenum Press.

WILSON, P. A. (1974). Entry of neutral amino acids into brain and other tissues. Ph.D. Thesis, University of London.