A Note on the Free Amino Acid Content of Rat Brain

By G. B. ANSELL and D. RICHTER

Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff

Some twenty different amino acids have been identified by paper chromatography in the free amino-nitrogen fraction of the brain (Roberts, Frankel & Harman, 1950; Astrup, Carlström & Stage, 1951; Walker, 1952). The quantitative values obtained by different investigators for the glutamine and glutamic acid contents show fairly good agreement (Krebs, Eggleston & Hems, 1949; Waelsch, 1949; Dawson, 1950): but few reliable figures are available for the concentrations of the other amino acids in fresh brain tissue. Awapara, Landua & Fuerst (1950) estimated the concentrations of four amino acids in the rat brain by extracting the ninhydrin-reacting spots from paper chromatograms. A number of free amino acids in rat brain have also been estimated by a microbiological method (Schurr, Thompson, Henderson, Williams & Elvehjem, 1950) and the values obtained were found to be affected by procedures such as exercising or cooling the animals before they were killed (Williams, Schurr & Elvehjem, 1950). Their control values were obtained on anaesthetized animals; but it appeared doubtful whether these values should be accepted as normal, since Dawson (1951) has found the glutamic acid level of the brain to be affected by thiopentone anaesthesia.

The present study started with the observation that there is a fairly rapid post-mortem liberation of amino acids in brain tissue incubated at a neutral pH, owing to the action of a proteinase that is active under these conditions (Ansell & Richter, 1954). It appeared that post-mortem changes of this kind might be a serious source of error in estimations of the amino acids of brain tissue; the pattern of amino-nitrogen distribution in the rat brain was therefore re-investigated in brain tissue rapidly frozen in liquid oxygen to minimize any post-mortem change. The possible effects of the method of killing and of anaesthesia on the brain amino acid content were investigated at the same time. The amino acids were estimated by the colorimetric method of Roberts & Frankel (1950) after a preliminary chromatographic separation.

EXPERIMENTAL

Young rats of about 50 g. (Wistar albino strain) were used throughout. Anaesthesia was induced by intraperitoneal injection of thiopentone ('Pentothal'; 60 mg./kg.), and the temperature of the anaesthetized animals was maintained by raising the environmental temperature and checking with a rectal thermometer. The rats were decapitated by a single sharp blow with a hammer on a razor placed above the neck and the heads frozen immediately by dropping into an insulated beaker containing liquid oxygen. Rats were also killed, where so stated, by dropping them whole into liquid oxygen. The frozen brains were dissected out without allowing them to thaw, crushed in a steel crusher, and the powder was dropped into a weighed centrifuge tube containing 5 ml. 12% (w/v) trichloroacetic acid at 0°. The tube was vigorously shaken, weighed and centrifuged. The total volume was taken as 5 ml. +0·8 ml. for each g. of brain. The solution was heated at 70° for 75 min. to convert glutamine into pyrrolidonecarboxylic acid and ammonia, and then stored at 4°.

Determination of total amino nitrogen. The total amino nitrogen was estimated by the colorimetric method of Moore & Stein (1948), the extinction coefficient being measured at 570 mμ.; glutamic acid was used as a standard. An ammonia standard was also used so that the total colour yield from the extract could be corrected for that due to ammonia, which was determined independently by the method of Conway (1939).

Paper chromatography. The tissue extracts were extracted twice with an equal volume of ether, which removed 95% of the trichloroacetic acid. They were then desalted by the method of Consden, Gordon & Martin (1947), care being taken to keep the extracts cool. The volume of extract used corresponded to 20–50 mg. fresh brain tissue, and the ninhydrin-reacting spots on two-dimensional phenol : collidine chromatograms (Dent, 1948) were identified by markers, by their stability to acid hydrolysis and by their reaction with $CuCO_3$ (Crumpler & Dent, 1949).

The quantitative estimation of the ninhydrin-reacting substances was carried out by the method of Roberts & Frankel (1950), after preliminary trials in which consistent results were obtained. Extinction coefficients were measured with an Ilford filter no. 812 (570 mμ.) because amino acids yield a colour having minimum transmission at this wavelength (Bull, Hahn & Baptist, 1949). It was found that although the Beer-Lambert law was obeyed over the range studied, the colour yield/μmole varied for individual amino compounds; there were also slight day-to-day variations. In each series of estimations control chromatograms of known amounts of amino compounds were therefore run concurrently and the amounts calculated by reference to the standards prepared at the same time.

RESULTS

The effect of the method of killing. In a series of preliminary experiments free amino nitrogen and amide nitrogen + ammonia (approximately equivalent to the glutamine) were determined in groups of

rats that had been killed by various different procedures. There is evidence that violent stimulation, such as is caused by decapitation, may affect the free ammonia and other metabolites in the brain (Richter & Dawson, 1948). The amino-nitrogen concentration did not appear to be greatly affected by the method of killing and no significant differences were observed, provided the tissue was frozen without delay (Table 1): but slightly lower amino-nitrogen values were noted in two groups of animals that had been anaesthetized before killing. Further experiments were therefore carried out to see if this difference was attributable to the anaesthesia or due to chance.

The effect of anaesthesia. Dawson (1951, 1953) reported that thiopentone anaesthesia reduces the glutamic acid level in the rat brain; but Williams *et al.* (1950) failed to observe any effect of anaesthesia on a number of other amino acids which they estimated in the brain. The question appeared to be worth pursuing, since some of the 'normal' figures

for the brain amino acids given in the literature were obtained on anaesthetized animals and an effect of anaesthetics on the nitrogen metabolism is not without interest. In the present study the total amino-nitrogen content of the brain in rats anaesthetized for various periods of time with thiopentone was compared with figures obtained under similar conditions for a series of unanaesthetized litter-mate controls (Table 2). Care was taken to maintain the temperature of the anaesthetized animals so that the results would be strictly comparable. The results showed a significantly lower amino-nitrogen content in the anaesthetized group ($P < 0.05$). However, the difference was small, amounting to only 2·5 mg. amino N/100 g. fresh brain. This was of the same order as the fall in the glutamic acid level in anaesthesia (about 2–3 mg. amino N/100 g. brain) observed by Dawson. It would appear from these figures that the effect is mainly or wholly accounted for by a fall in the glutamic acid level.

Table 1. *The effect of the method of killing on free amino nitrogen and (amide nitrogen + ammonia nitrogen) in rat brain*

The figures give mean values for duplicate determinations on the number of animals indicated. The anaesthetized animals received 60 mg. thiopentone/kg. body wt.

No. of rats	Method of killing	Amino N (mg./100 g. brain)	S.E.M.	Amide N + ammonia N (mg./100 g. brain)	S.E.M.
3	Decapitated and head frozen immediately	42·2	±1·57	7·5	±0·49
4	Animal frozen whole	43·4	±1·58	9·4	±0·38
7	Anaesthetized and frozen whole	41·2	±1·55	7·4	±0·51
6	Anaesthetized, decapitated and head frozen immediately	39·8	±1·17	9·8	±0·45

Table 2. *The effect of anaesthesia on free amino nitrogen and (amide nitrogen + ammonia nitrogen) in rat brain*

The animals were killed by decapitation and the heads immediately frozen in liquid oxygen. The anaesthetized animals received 60 mg. thiopentone/kg. body wt. The figures give the means of duplicate determinations.

		mg./100 g. fresh brain			
		Unanaesthetized litter-mate controls		Anaesthetized	
Rat no.	Duration of anaesthesia (min.)	Amino N	(Amide N + ammonia N)	Amino N	(Amide N + ammonia N)
1–2	72	43·8	8·5	44·9	10·0
3–4	77	41·4	8·5	38·6	9·6
5–6	104	41·0	9·0	40·9	10·8
7–8	77	45·7	9·1	35·3	8·8
9–10	95	37·3	8·4	38·3	9·1
11–12	45	42·2	8·8	40·7	10·5
13–14	55	44·5	7·7	41·2	10·7
15–16	60	48·2	8·0	41·0	8·8
17–18	60	44·0	8·5	41·0	8·6
19–20	57	43·3	8·8	42·5	8·8
21–22	60	38·3	7·8	37·8	8·4
	Mean	42·7	8·5	40·2	9·5
	S.E.M.	±0·90	±0·14	±0·75	±0·26

Table 3. *Concentrations (mg. amino N/100 g. fresh brain) of amino acids and related compounds in rat brain*

The animals included in brackets were litter mates. The figures for total amino N exclude glutamine.

Rat no.	Glutamic acid	Aspartic acid	Glutathione*	O-Phosphoryl-ethanolamine	Glycine	Serine	Taurine	Alanine	γ-Amino-butyric acid	Total amino N
1	11·8	4·1	—	2·3	1·6	1·5	5·4	0·9	3·1	41·7
2	12·0	4·5	—	2·2	2·2	1·5	6·4	1·3	3·8	45·9
3	14·1	3·9	2·4	1·9	1·5	1·4	8·8	0·6	2·8	44·4
4	14·6	3·3	2·5	1·7	1·7	1·6	7·8	0·7	2·3	45·2
5	13·7	3·3	2·4	2·7	1·9	(0·7)	9·3	0·4	2·2	43·4
Mean	13·2	3·8	2·4	2·2	1·8	1·5	7·5	0·8	2·8	44·1
Other workers	12·9† 15·0§	5·1†	—	1·6†	—	—	3·3†	—	1·6‡	—

* Observed only on chromatograms treated with hydrogen peroxide and ammonium molybdate (Dent, 1948).
† Awapara, Landua & Fuerst (1950).
‡ Awapara, Landua, Fuerst & Seale (1950).
§ Dawson (1950).

The figures also showed a small but significant rise in the amide-nitrogen (glutamine) fraction in anaesthesia $(P < 0·01)$ (cf. Dawson, 1951). The effects of thiopentone anaesthesia on the amino nitrogen and amide nitrogen were confirmed qualitatively by the visual comparison of four pairs of chromatograms prepared from desalted brain extracts of normal and anaesthetized rats. A careful comparison showed in every case a slight fall in the intensity of the glutamic acid spot and an apparent slight increase in the intensity of the spots for glutamine and γ-aminobutyric acid. The intensity of the other spots remained unchanged as far as could be judged by this method; but Dawson (1953) has recently shown that the aspartic acid content is increased in thiopentone anaesthesia. The present results therefore confirm Dawson's finding of a fall in the glutamic acid level and a rise in the glutamine in anaesthesia; they also agree with the implication of Williams *et al.* (1950) that certain other amino acids are not significantly affected.

The concentration of individual amino acids in brain tissue. The values obtained for the concentration of nine amino acids and related compounds, and for the total amino nitrogen in fresh rat brain are given in Table 3. Each figure represents the mean of two determinations on the same trichloroacetic acid extract prepared from frozen whole brain tissue. The rats were killed by decapitation and the heads immediately frozen in liquid oxygen.

Glutamine was converted into pyrrolidone-carboxylic acid and was not determined individually or included in the figure for the total amino nitrogen. If the glutamine amino-nitrogen content of rat brain is taken as 7 mg./100 g. tissue, then the total free amino nitrogen is 49·7 mg./100 g. (using the mean of the 'normal' values in Table 2). This figure agrees approximately with the figures of 52 mg. reported by Awapara, Landua & Fuerst (1950) and 50·5 mg. which can be calculated from the results of

Dawson (1950). It may be seen that the three related compounds glutamine, glutamic acid and γ-aminobutyric acid accounted for 45 % of the total amino nitrogen.

The figures for the individual amino compounds are of the same order as those reported by Awapara, Landua & Fuerst (1950) and Awapara, Landua, Fuerst & Seale (1950) for the five compounds estimated by them, except for taurine which was found at about double the concentration they gave. However, the present figure obtained for taurine would agree with the qualitative observations of Roberts *et al.* (1950) and of Boulanger & Biserte (1951), who reported that it was present in relatively high concentration. The spots due to threonine and cysteic acid (derived from cyst(e)ine and observed on chromatograms treated with hydrogen peroxide and ammonium molybdate (Dent, 1948)) were too faint for quantitative estimation by the present methods and their intensification by using a larger quantity of extract was prohibited, since it would have led to errors due to the overlapping with other spots.

DISCUSSION

The amino acids of the brain may be regarded as falling into two main groups. There is the large group of amino acids present in relatively low concentration corresponding to the amino acid 'pool' in other tissues: these are presumably concerned in the processes of synthesis and breakdown of the tissue proteins, which are constantly taking place. There is, secondly, the small group of related compounds, glutamine, glutamic acid and γ-amino-butyric acid, which are present in relatively high concentrations and to which a special function must be ascribed. Glutamic acid is unique among the amino acids of the brain in its ability to undergo oxidation, amidation, transamination and decarboxylation, and it appears to occupy a central position in the metabolism of the brain.

The nine amino compounds estimated individually in the present study corresponded altogether to 36 mg. amino N/100 g. brain. If 7 mg./100 g. brain is taken for the glutamine amino nitrogen and 3 mg./100 g. brain for the group of twelve less abundant amino acids (including threonine) estimated microbiologically by Schurr, Thompson, Henderson, Williams & Elvehjem (1950), this adds up to 46 mg./100 g. brain, which accounts for 92·5 % of the total amino nitrogen. In the remaining 7·5 % will occur a number of other amino compounds present in too small a concentration to give satisfactory spots under these conditions on chromatograms.

Roberts *et al.* (1950) reported an acid-labile spot X appearing 'underneath' glutamic acid (also designated as 'O' in a later paper by Roberts & Frankel (1950)) on phenol : collidine chromatograms of ethanolic extracts of mouse brain: a spot 'under glutamic acid' was also mentioned by Awapara, Landua & Fuerst (1950). This unidentified spot was presumably *O*-phosphorylethanolamine, which has now been identified in rat brain extracts (Ansell & Dawson, 1951).

Roberts *et al.* (1950) also reported a further substance 'X' in mouse brain extracts which disappeared on acid hydrolysis, (designated 'P' in a later paper by Roberts & Frankel, 1950). This substance scarcely moved in phenol and 'collidine' runs and in this respect resembles hydroxylysine phosphate, which was identified in calf embryo muscle by Gordon (1949) and reported by Astrup *et al.* (1951) to be present in ox brain extracts. A spot in this position was never observed on chromatograms of desalted extracts prepared from rat brain frozen in liquid oxygen, though a ninhydrin-reacting streak was sometimes seen on chromatograms of undesalted extracts. Astrup *et al.* do not state how soon after death their extracts were prepared: but they used for their chromatograms an amount of extract equivalent to about 10 times the amount of brain tissue used in the present study, and it is therefore very likely that such a spot would not be seen under our conditions. These workers also reported the presence of a small amount of β-alanine and carnosine in ox brain extracts.

The only peptide estimated in the present study was glutathione, but other peptides are also present in rat brain tissue, in an amount corresponding to about 20 mg. peptide-bond nitrogen/100 g. of brain (Ansell & Richter, 1954). The results of Schurr, Thompson, Henderson & Elvehjem (1950) and of Boulanger & Biserte (1951) indicate that peptides of leucine, isoleucine, valine, histidine, proline, alanine, glycine, aspartic acid and γ-aminobutyric acid are present in brain tissue.

SUMMARY

1. The free amino-nitrogen content of rat brain falls by about 2·6 mg./100 g. tissue in thiopentone ('Pentothal') anaesthesia. This is attributable mainly to a fall in the glutamic acid level.

2. Nine amino compounds visible on paper chromatograms were estimated in deproteinized extracts of rat brain which had been frozen in liquid oxygen immediately after death.

REFERENCES

Ansell, G. B. & Dawson, R. M. C. (1951). *Biochem. J.* **50**, 241.

Ansell, G. B. & Richter, D. (1954). *Biochim. biophys. Acta* **13**, 92.

Astrup, T., Carlström, G. & Stage, A. (1951). *Acta physiol. scand.* **24**, 202.

Awapara, J., Landua, A. J. & Fuerst, R. (1950). *Biochim. biophys. Acta*, **5**, 457.

Awapara, J., Landua, A. J., Fuerst, R. & Seale, B. (1950). *J. biol. Chem.* **187**, 35.

Boulanger, P. & Biserte, G. (1951). *C.R. Acad. Sci., Paris*, **233**, 1498.

Bull, H. B., Hahn, J. W. & Baptist, V. H. (1949). *J. Amer. chem. Soc.* **71**, 550.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.

Conway, E. J. (1939). *Microdiffusion Analysis and Volumetric Error.* London: Crosby, Lockwood and Son.

Crumpler, H. R. & Dent, C. E. (1949). *Nature, Lond.*, **164**, 441.

Dawson, R. M. C. (1950). *Biochem. J.* **47**, 386.

Dawson, R. M. C. (1951). *Biochem. J.* **49**, 138.

Dawson, R. M. C. (1953). *Biochim. biophys. Acta*, **11**, 548.

Dent, C. E. (1948). *Biochem. J.* **43**, 169.

Gordon, A. H. (1949). *Biochem. J.* **45**, 99.

Krebs, H. A., Eggleston, L. V. & Hems, R. (1949). *Biochem. J.* **44**, 159.

Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.

Richter, D. & Dawson, R. M. C. (1948). *J. biol. Chem.* **176**, 1199.

Roberts, E. & Frankel, S. (1950). *J. biol. Chem.* **187**, 55.

Roberts, E., Frankel, S. & Harman, P. J. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 383.

Schurr, P. E., Thompson, H. T., Henderson, L. M. & Elvehjem, C. A. (1950). *J. biol. Chem.* **182**, 29.

Schurr, P. E., Thompson, H. T., Henderson, L. M., Williams, J. N. jun. & Elvehjem, C. A. (1950). *J. biol. Chem.* **182**, 39.

Waelsch, H. (1949). *Lancet*, **2**, 1.

Walker, D. M. (1952). *Biochem. J.* **52**, 679.

Williams, J. N. jun., Schurr, P. E. & Elvehjem, C. A. (1950). *J. biol. Chem.* **182**, 55.