Effect of Thyroid Hormone on Metabolic Compartmentation in the Developing Rat Brain

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1. The effects of treatment with thyroid hormone (tri-iodothyronine) and of neonatal thyroidectomy on the cerebral metabolism of [U - ^{14}C]leucine were investigated during the period of functional maturation of the rat brain extending from 9 to 25 days after birth. 2. Age-dependent changes in the labelling of brain constituents under normal conditions appear to depend on changes in the availability of blood-borne [^{14}C]leucine resulting from differential rates of growth of body and brain; but developmental changes in the pool size of free leucine and in the rates of protein synthesis and oxidation of leucine are also involved. 3. Treatment with thyroid hormone had no significant effect on the conversion of leucine carbon into proteins and lipids; and the age-dependent changes in the concentration and specific radioactivity of leucine were similar to controls. On the other hand there was an acceleration in the conversion of leucine carbon into amino acids associated with the tricarboxylic acid cycle. These observations indicate that leucine oxidation was the process mainly affected. 4. The specific radioactivity of glutamine relative to that of glutamate was used as an index of metabolic compartmentation in brain tissue. Treatment with thyroid hormone advanced the development of metabolic compartmentation. 5. Neonatal thyroidectomy led to a marked decrease in the conversion of leucine carbon into proteins and lipids and to a significant increase in the amount of ^{14}C combined in the amino acids associated with the tricarboxylic acid cycle. The age-dependent increase in the glutamate/glutamine specific-radioactivity ratio was strongly retarded. 6. The increased conversion of leucine carbon into cerebral amino acids applied to glutamate and aspartate, but not to glutamine and γ -aminobutyrate. This observation facilitated the understanding of the effects of thyroid deprivation on brain metabolism and provided new evidence for the allocation of morphological structures to the metabolic compartments in brain tissue. 7. In contrast with the marked effects of the thyroid state on metabolic compartmentation, it had relatively little effect on the developmental changes in the concentration of amino acids in the brain. 8. The rate of conversion of leucine carbon into the 'cycle amino acids' both under normal conditions and in thyroid deficiency indicated a special metabolic relationship between glutamate and aspartate on the one hand, and glutamine and γ -aminobutyrate on the other.

Thyroid hormone has marked effects on the development of the mammalian central nervous system (reviews by Eayrs, 1968; Balázs, 1970). Thyroid deficiency in early life gives rise to severe and frequently irreversible behavioural changes associated with structural changes in the cortical neurons, hypoplasia of the neuropil (Eayrs, 1968) and retardation of myelination (Balázs, Brooksbank, Davison, Eayrs & Wilson, 1969; Walravens & Chase, 1969). Conversely, treatment with thyroid hormone in infancy results in an advancement of the morphological and functional

maturation as indicated by the development of electrocortical activity, innately organized behaviour and the formation of dendritic spines on the cortical neurons (Eayrs, 1968; Schapiro, 1968). The biochemical maturation of the brain is also affected (reviews by Hamburgh, 1968; Balázs, 1970), as shown by an advancement by thyroid treatment and a retardation in thyroid deficiency of the maturation of the energy metabolism of the brain (Cocks, Balázs, Johnson & Eayrs, 1970). In the present investigation the effects of thyroid state on the biochemical maturation were studied

by using another 'maturation index', the development of the metabolic heterogeneity of brain tissue (Patel & Balázs, 1970). The results provide new evidence of the relation of metabolic compartmentation to morphological structures in the brain. A preliminary account of this work has been given (Patel, Balázs & Richter, 1969).

EXPERIMENTAL

Animals. Newborn male albino rats were divided into experimental and control groups as described by Balázs, Kovács, Teichgräber, Cocks & Eayrs (1968). (a) Treatment with thyroid hormone: rats of the Sprague-Dawley strain were used. The experimental animals received a subcutaneous injection of 3,3',5-tri-iodo-L-thyronine (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.): 25 µg at birth, and 0.5–1.0 µg on alternate days thereafter until the end of the experiment. If the animals showed excessive tremor or loss of body weight one injection was omitted. The control animals received the same volume (50 µl) of physiological saline (0.9% NaCl). (b) Thyroid deficiency: rats of the Porton strain were used in these studies. Thyroidectomy was performed on the day of birth by a subcutaneous injection of 150 µCi of ¹³¹I. The control animals received the same volume (100 µl) of physiological saline. The development of metabolic compartmentation and the age-dependent changes in leucine metabolism were similar in the Porton strain and the Sprague-Dawley strain of rats.

Littermate control and experimental animals received a subcutaneous injection of 10 µCi of [U-¹⁴C]leucine/100 g body wt. (specific radioactivity 31 mCi/mmol). The animals were killed by immersion in liquid N₂ at 10 min after the injection, with the exception of the 12-day-old rats (thyroid-deficiency series) which were killed at 20 min.

Treatment of tissues. Fractions containing acid-soluble, lipid, nucleic acid and protein constituents were separated by the method described by Patel & Balázs (1970). The brains (cerebrum) were homogenized in 0.4 M-HClO₄ and the residues washed with 0.2 M-HClO₄. Lipids were extracted with consecutive treatments with ethanol saturated with sodium acetate, chloroform-methanol (2:1, v/v) and ether. The lipids were further purified by the method of Folch, Lees & Sloane-Stanley (1957). The nucleic acids were extracted with 5% trichloroacetic acid at 90°C for 15 min.

Fractionation of the acid-soluble extract. This was done by using the technique of Minard & Mushahwar (1966) with modifications as described by Patel & Balázs (1970). Briefly, glutamate, aspartate and free carboxylic acids were separated from the acid-soluble extract by using an anion-exchange resin (Dowex 1, acetate form). Glutamine and acetyl-CoA derivatives contained in the effluent were hydrolysed with 4 M-HCl for 2 h at 100°C, and the resulting glutamate and carboxylic acids were adsorbed on a column of Dowex 1 (acetate form) resin. A fraction containing neutral compounds including glucose was isolated from the effluent of the second column by passing the samples through a cation-exchange resin (Dowex 50, H⁺ form). The elution of glutamate and aspartate and the further purification of aspartate was done as described

before. The carboxylic acids were stripped from both columns of Dowex 1 resin after the amino acids had been eluted; the pooled fractions are designated in Table 1 as 'carboxylic acids' fraction. Alanine, leucine and γ-aminobutyrate were analysed by using a Technicon amino acid analyser. The sequential elution of the amino acids was effected as described by Hamilton (1963) but the pH of the last citrate buffer was 5.28, which ensured that the γ-aminobutyrate peak was pure.

The technique in the experimental series dealing with the effects of thyroid deficiency was slightly modified. At first, the non-amino acid constituents of the neutralized acid-soluble fraction were separated from the amino acids by passing the extract through a column (0.55 cm × 20 cm) of cation-exchange resin (Dowex 50; X8; H⁺ form). The resin was washed with 40 ml of water; thus phosphoethanolamine was collected together with neutral compounds and carboxylic acids in the effluent. Next the amino acids were eluted from the resin by using 60 ml of 1 M-NH₃. The eluate was evaporated under reduced pressure at 40°C; the dry residue was dissolved in a small volume of water, and glutamate, aspartate and glutamine were separated from the other amino acids by using two consecutive columns of Dowex 1 (acetate form) resin as described above. The rest of the amino acids were separated by using a Technicon amino acid analyser with the modified elution programme.

Analytical methods. The amino acids, glutamate, aspartate and glutamine, were determined by the ninhydrin procedure in a Technicon AutoAnalyzer by the sample plate technique. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Determination of radioactivity. Radioactivities of the samples were determined in a Nuclear-Chicago Scintillation Spectrometer. The counting fluid contained 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene-2-ethoxyethanol (7:3, v/v), and 2-ethoxyethanol was used to ensure a one-phase system. The homogenate of whole tissue and the protein fraction were dissolved in 1 M-Hyamine hydroxide in methanol [Nuclear Enterprises (G.B.) Ltd., Edinburgh 11, U.K.] and lipids in Nuclear-Chicago solubilizer. Quenching was corrected by the channels-ratio technique.

Statistical analysis. The results were analysed statistically by using analysis of variance with a two-way classification (age versus treatment) (Duncan, 1955). When a significant interaction that obscured the main effects occurred in this design, the results were reanalysed by using a randomized-block design. The standard errors were calculated from the residual mean square in the appropriate way.

RESULTS

Fate of [U-¹⁴C]leucine in the developing rat brain. In previous studies it was found that [U-¹⁴C]-leucine is a suitable substrate to study the development of metabolic compartmentation in the rat brain (Patel & Balázs, 1970). [U-¹⁴C]Leucine was used therefore in the present work to investigate the effects of the thyroid state on the compartmentation of glutamate during the period when compartmentation develops rapidly from 9 to 25

Fig. 1. Fate of [U-¹⁴C]leucine in brain tissue during development. Rats were killed at 10 min after a subcutaneous injection of [U-¹⁴C]leucine (10 μ Ci/100 g body wt.). The curves were constructed by using the normal data obtained in three independent series of experiments; for the statistical evaluations see the experimental series on tri-iodothyronine-treated and thyroid-deficient rats respectively in the present paper and also the results given by Patel & Balázs (1970). (a) ¹⁴C content of whole tissue (O) and of proteins (Δ); ■, specific radioactivity of free leucine; ●, concentration of free leucine. (b) ¹⁴C content of amino acids associated with the tricarboxylic acid cycle (□) and of lipids (▽); ×, specific radioactivity of 'cycle' amino acids (i.e. the sum of glutamate, glutamine, aspartate and γ -aminobutyrate) relative to that of leucine.

days after birth. The fate of leucine carbon in the brain was investigated at 10 min after injection. The pattern of ¹⁴C distribution in the brain of normal animals is summarized in Fig. 1 on the basis of three independent experimental series. The results show that the cerebral metabolism of [U-¹⁴C]leucine changes during development. The ¹⁴C content of the tissue increased significantly between 9 and 12 days, and it decreased after the age of about 18 days. The age-curve of total ¹⁴C reflected more or less that of the specific radioactivity of free leucine and was paralleled by the age-curve of the ¹⁴C content of proteins. The significance of protein synthesis in the metabolism of leucine was indicated by the observation that about 50% of the ¹⁴C was combined in proteins from the age of 12 days (Tables 1 and 2). The proportion of ¹⁴C in proteins was much less, 30%, at 9 days after birth. Catabolism is the other main route of leucine metabolism. The proportion of acid-soluble ¹⁴C combined in the immediate products of leucine metabolism ('carboxylic acid' fraction; Table 1) did not change much during development (Patel & Balázs, 1970). On the other hand, the proportion of ¹⁴C in the amino acids associated with the tricarboxylic acid cycle increased markedly during development. The ¹⁴C content of the amino acids per unit weight increased

from 9 through 21 days after birth and the values decreased later (Fig. 1). Blood-borne glucose was not significantly involved in the labelling of these amino acids: the ¹⁴C content of the fraction containing glucose was low (Table 1), and the specific radioactivity of alanine relative to that of glutamate was only about 0.1 during the whole experimental period (Fig. 2). Thus, the ¹⁴C content of the amino acids associated with the tricarboxylic acid cycle may be taken to reflect the amount of leucine carbon that is oxidized in the brain. A closer approximation of the rate of leucine oxidation may be obtained by expressing the specific radioactivity of the amino acids in terms of that of leucine. These values increased substantially during the period from 9 to 12 days of age, and did not change much later. The conversion of leucine carbon into lipids was small during the whole experimental period, but the increase in the concentration of ¹⁴C-labelled lipids was considerable, about ninefold (Fig. 1).

Effects of treatment with tri-iodothyronine on leucine metabolism in the brain. Treatment with tri-iodothyronine did not affect significantly the age-curve for the specific radioactivity of leucine in the brain. The overall pattern of distribution of ¹⁴C was also similar to that of the controls, with the exception of the conversion of leucine carbon

Table 1. *Effect of treatment with thyroid hormone on the conversion of leucine carbon into brain constituents during maturation*

Rats were given tri-iodothyronine from birth, as described in the Experimental section. Littermate controls (N) and the experimental animals (T ₃) (three animals each at every age-point studied) received a subcutaneous injection of [U- ¹⁴ C]leucine (10 μCi/100g body wt.) and they were killed by immersion into liquid N ₂ at 10min after the injection. The cerebrum of each rat was processed separately as described in the Experimental section. s.e., Standard error; N.S., no significant difference compared with controls (P>0.05). The numerical values in the columns of significance refer to P.																		
Experimental treatment	Age (days) ...			9			12			15			21			Significance		
	T ₃	N		T ₃	N		T ₃	N		T ₃	N		T ₃	N		'Age'	s.e.	'Treat- ment'
Whole tissue (d.p.m./g of tissue)	69.465	64.416		86.933	100.012		98.820	92.987		88.718	94.861		88.718	94.861		<0.01	6258	N.S.
Recovery of ¹⁴ C in main fractions as a percentage of ¹⁴ C in whole tissue	92.24	96.39		92.31	91.64		93.68	93.22		93.51	90.63		93.51	90.63		N.S.	1.37	N.S.
Distribution of ¹⁴ C in the main fractions:																		
Protein fraction (%)*	37.17	29.12		38.17	44.52		42.97	47.44		51.34	52.69		51.34	52.69		<0.01	1.26	N.S.
(d.p.m./g)	23.647	18.036		30.623	40.823		39.765	41.112		42.582	45.045		42.582	45.045		<0.01	2640	N.S.
Lipid fraction (%)*	0.30	0.31		1.07	1.01		1.28	1.18		2.25	2.08		2.25	2.08		<0.01	0.16	N.S.
(d.p.m./g)	190	191		860	916		1192	1014		1861	1719		1861	1719		<0.01	107	N.S.
Acid-soluble fraction (%)*	62.54	70.57		60.76	54.48		55.75	51.38		46.40	45.23		46.40	45.23		<0.01	1.28	N.S.
Acid-soluble fraction (d.p.m./g of tissue)	40.303	43.882		48.786	49.816		51.635	44.461		38.498	38.836		38.498	38.836		<0.05	3149	N.S.
Distribution of ¹⁴ C as a per-centage of ¹⁴ C in the acid-soluble fraction:																		
(a) Leucine†	65.89	73.24		56.02	59.02		45.27	53.38		54.66	52.88		54.66	52.88		<0.01	4.01	N.S.
(b) 'Carboxylic acids' fraction‡	14.59	17.84		17.97	16.78		16.64	18.35		11.98	11.37		11.98	11.37		N.S.	3.27	N.S.
(c) Amino acids associated with the tricarboxylic acid cycle§	4.80	3.33		13.71	12.58		21.43	15.44		25.32	23.19		25.32	23.19		<0.01	1.60	<0.05
(d) 'Glucose' fraction	0.21	0.14		0.21	0.12		0.23	0.22		0.35	0.23		0.35	0.23		N.S.	0.06	N.S.

Fig. 2. Effect of treatment with thyroid hormone during postnatal development on the specific radioactivity of amino acids associated with the tricarboxylic acid cycle. Experimental details were given in Table 1. The values for the specific radioactivities were adjusted to an arbitrary base of 1×10^5 d.p.m./g of fresh tissue. The mean values are plotted. Δ and $\blacktriangle$, Glutamine; $\circ$ and $\bullet$, γ -aminobutyrate; $\diamond$ and $\blacklozenge$, alanine; ∇ and $\blacktriangledown$, glutamate; $\square$ and $\blacksquare$, aspartate; $\circ, \Delta, \square, \nabla, \diamond$, animals treated with tri-iodothyronine from birth; $\bullet, \blacktriangle, \blacksquare, \blacktriangledown, \blacklozenge$, control animals.

	Glutamine	γ -Amino- butyrate	Alanine	Glutamate	Aspartate
S.E.	75	0.09	10	47	38
Significance:					
'Age'	<0.01	<0.01	N.S.	<0.01	<0.01
'Treatment'	<0.01	N.S.	N.S.	N.S.	<0.05

N.S. under the heading 'Significance' stands for no significant difference ($P > 0.05$) and the numerical values refer to P . The γ -aminobutyrate results were analysed after using a logarithmic transformation to stabilize the variances of the individual groups. The s.e. quoted applies to the logarithmic transformation of the means ($\times 10$) and not to the means shown in the figure.

into the amino acids associated with the tricarboxylic acid cycle, which was consistently higher than in controls (Table 1).

Incorporation of ^{14}C into amino acids. Since treatment with tri-iodothyronine appeared to affect the oxidation of leucine rather than the other reactions of leucine metabolism, the amino acids associated with the tricarboxylic acid cycle were analysed in detail.

A high proportion of the ^{14}C in the amino acids was combined in glutamate during brain development; 67% at 9 days, about 50% at 12–15 days and 44% at 35 days after birth. The proportion of ^{14}C in glutamine was 20% at 9 days, but it increased progressively and at 35 days glutamine contained the same amount of ^{14}C as glutamate. The other

amino acids in decreasing order of ^{14}C content were aspartate, γ -aminobutyrate and alanine. It is known that the concentration of amino acids changes during brain maturation (cf. Agrawal, Davis & Himwich, 1966, and Table 5). The incorporation of ^{14}C into the amino acids was therefore expressed in terms of specific radioactivities. The specific radioactivity of glutamate increased from 159 d.p.m./ μmol at 9 days to 465 (s.e. ± 49) d.p.m./ μmol at 12 days after birth, and the values did not change much later. Since the total ^{14}C concentration in the tissue changed during development, for the sake of comparison the results were adjusted to an arbitrary base of 1×10^5 d.p.m./g fresh wt. The age-curves in Fig. 2 showed that there was a sharp rise in the incorporation rate of ^{14}C into the amino

acids after the age of 9 days, with the exception of alanine (cf. above). The specific radioactivity of glutamine increased progressively during the whole experimental period, whereas that of glutamate reached a plateau at 12 days after birth. In this respect the incorporation of ^{14}C into γ -aminobutyrate followed that into glutamine, which was in marked contrast with the behaviour of aspartate, which paralleled that of glutamate.

Treatment with tri-iodothyronine did not affect significantly the pattern of incorporation of ^{14}C into the amino acids associated with the tricarboxylic acid cycle. The treatment led, however, to a quantitative increase in the incorporation rate that was significant during the whole period in the case of glutamine and aspartate (Fig. 2).

The evidence summarized by Patel & Balázs (1970) indicated that the pathway of leucine catabolism in brain is similar to that observed in other tissues (Meister, 1965). The breakdown of $[\text{U-}^{14}\text{C}]$ -leucine leads to the formation of $[\text{U-}^{14}\text{C}]$ -acetyl-CoA; thus the amino acid that is first labelled as a result of the oxidation of $[\text{U-}^{14}\text{C}]$ -leucine is glutamate. To decrease the variation in the labelling of constituents as a result of differences in the uptake of $[\text{U-}^{14}\text{C}]$ -leucine by brain tissue the specific radioactivities of the amino acids were expressed in Fig. 3 relative to that of glutamate. When the effects of tri-iodothyronine treatment were considered the main difference compared with controls was the consistent increase in the relative specific radioactivity of glutamine. The period studied in the present work was characterized in the brain of normal rats by the development of metabolic heterogeneity; the glutamine/glutamate specific radioactivity ratio increased during this period, and reached values in excess of 1. Treatment with thyroid hormone in infancy accelerated the developmental processes resulting in an advancement of 1–3 days.

Effect of neonatal thyroidectomy on the fate of $[\text{U-}^{14}\text{C}]$ -leucine in the developing brain. Neonatal thyroidectomy had a marked effect on the distribution of leucine carbon in the major constituents of the tissue (Table 2); there was a significant decrease in the amount of ^{14}C incorporated into proteins and lipids, when compared with controls. The acid-soluble fraction, on the other hand, contained more ^{14}C both in absolute and relative terms; this resulted from an increase in the proportion of ^{14}C in both free leucine and the amino acids associated with the tricarboxylic acid cycle. The increase in the ^{14}C content of the 'cycle-amino acids' was especially high, about twice the control value in the brain of 25-day-old rats. The incorporation of ^{14}C into the amino acids was therefore investigated. For sake of comparison (cf. above, Fig. 2) the specific radioactivities were expressed in terms of a constant ^{14}C content/g of fresh tissue (Table 3).

Fig. 3. Specific radioactivity of amino acids relative to that of glutamate: (a) glutamine; (b) aspartate; (c) γ -aminobutyrate. The results were taken from three independent experimental series; $\circ$, Patel & Balázs (1970), and from the present work. Tri-iodothyronine-treatment: Δ , controls; $\blacktriangle$, experimental animals. Neonatal thyroidectomy: $\square$, controls; $\blacksquare$, experimental animals. Both treatments led to significant differences compared with normal in (a) ($P < 0.01$); no significant effects of thyroid state in (b); and in (c) thyroid deficiency resulted in a significant difference ($P < 0.01$), whereas the effect of treatment with tri-iodothyronine was not significant.

The results indicated that thyroid deficiency did not lead to a uniform rise in the labelling of the amino acids associated with the tricarboxylic acid cycle. The specific radioactivities of glutamate and aspartate were markedly increased, but those of glutamine and γ -aminobutyrate were not significantly different from the control values.

Effect of thyroid deficiency on free leucine. The observation that more leucine carbon was converted into the amino acids associated with the tricarboxylic acid cycle in the brain of hypothyroid

Table 2. *Effect of neonatal thyroidectomy on the conversion of leucine carbon into brain constituents during maturation*

Rats were thyroidectomized by a subcutaneous injection of $150 \mu\text{Ci}$ of ^{131}I at birth. Three littermate controls (normal) and three experimental animals (T_x) of the ages specified in the Table received a subcutaneous injection of $[\text{U-}^{14}\text{C}]\text{leucine}$ ($10 \mu\text{Ci}/100 \text{g}$ body wt.); the animals were killed by immersion into liquid N_2 at 10 min after the injection, except the 12-day-old rats which were killed at 20 min(*). In the column 'Significance' N.S. stands for no significant difference (i.e. $P > 0.05$) and the numerical values refer to P . The results for the 12-day-old group (killed at 20 min after injection) were not included in the statistical analysis given in the Tables (except Table 5b).

Experimental treatment	Age (days) ...				18				25				Significance	
					12*								Age	Treatment
					T_x	Normal	T_x	Normal	T_x	Normal	T_x	Normal		
Whole tissue (d.p.m./g of tissue)					99587	87770	91022	110967	90972	73516	7942		<0.05	N.S.
Recovery of ^{14}C in main fractions as a percentage of ^{14}C in whole tissue					96.00	96.02	92.91	92.09	97.69	95.73	8.13		N.S.	N.S.
Distribution of ^{14}C in the main fractions:														
Protein fraction (%)†					63.12	76.22	35.29	52.91	34.00	46.21	3.81		N.S.	<0.01
Lipid fraction (d.p.m./g) (%)†					60521	64036	31378	54014	29513	32214	5548		N.S.	<0.05
Acid-soluble fraction (d.p.m./g) (%)†					—	—	1.00	1.49	1.17	2.10	0.11		<0.01	<0.01
(a) Leucine (d.p.m./g) (%)†					36.90	23.78	63.70	45.60	64.84	51.70	3.86		N.S.	<0.01
(b) Amino acids associated with the tricarboxylic acid cycle† (d.p.m./g) (%)†					34997	20083	52676	46600	56106	36144	3445		N.S.	<0.01
					—	—	38.32	26.50	30.73	28.25	2.90		N.S.	<0.05
					—	—	34081	29451	28198	20648	3063		<0.05	N.S.
					16.96	15.30	10.21	7.56	16.14	9.47	1.19		<0.05	<0.01
					16803	13312	9353	8395	14305	6904	884		N.S.	<0.01

† The distribution of ^{14}C was expressed as the percentage of ^{14}C recovered in the main fractions (protein, lipid and acid-soluble fractions).

‡ The amino acids were glutamate, glutamine, aspartate, alanine and γ -aminobutyrate.

Table 3. *Specific radioactivity of amino acids associated with the tricarboxylic acid cycle in the brain of thyroid-deficient and normal rats after the administration of [U-¹⁴C]leucine*

Experimental details were given in Table 2. The specific radioactivities were adjusted to an arbitrary base of 1×10^5 total d.p.m./g of tissue wet wt. The mean values are given and the changes as a result of neonatal thyroidectomy are also expressed in percentage of the control values ($T_x/N \times 100$). Under the heading 'Significance' N.S. stands for no significant difference (i.e. $P > 0.05$), and the numerical values refer to P .

Age (days)	Experimental treatment	Adjusted specific radioactivity				
		Glutamate	Glutamine	Aspartate	γ -Amino-butyrate	Alanine
12*	Thyroidectomy	1565	1578	969	—	—
	Normal	1292	1485	711	—	—
	$T_x/N \times 100$	121	106	136	—	—
18	Thyroidectomy	675	978	346	344	101
	Normal	466	837	300	313	62
	$T_x/N \times 100$	145	117	115	110	163
25	Thyroidectomy	956	1120	593	291	94
	Normal	447	990	301	262	82
	$T_x/N \times 100$	214	113	197	111	115
S.E.		30	93	29	41	16
Significance:						
'Age'		N.S.	N.S.	N.S.	N.S.	N.S.
'Treatment'		<0.01	N.S.	<0.01	N.S.	N.S.

* The 12-day-old rats were killed at 20 min after the injection of [U-¹⁴C]leucine; the other animals were killed at 10 min.

animals compared with controls may be related to an effect of thyroid deficiency on the pool size and ¹⁴C content of free leucine.

Table 5 shows that the concentration of free leucine was not significantly affected during the period 18–25 days after birth by neonatal thyroidectomy. The specific radioactivity of leucine was, however, 30% higher than in controls at 25 days of age (Table 4). The conversion of leucine carbon into both the amino acids and proteins was therefore expressed in terms of the specific radioactivity of free leucine. The results indicated that in the normal animals the rate of leucine oxidation did not change much during the period studied (cf. the values for the 'cycle-amino acids' in Table 4), but the incorporation rate of leucine into proteins decreased significantly.

The major effects of thyroid deficiency on brain metabolism were as follows: when compared with normal animals the rate of ¹⁴C incorporation into protein was decreased by 30% at 18 days and by 20% at 25 days after birth. On the other hand, more leucine carbon passed through the tricarboxylic acid cycle (cf. values for 'cycle-amino acids' in Table 4). A change in the processes of leucine oxidation characteristic of the normal brain was also indicated by the results; in the brain of 25-day-old hypothyroid rats the specific radioactivity of both glutamate and aspartate relative to that of leucine was about 100% higher than in controls,

whereas the values for glutamine and γ -aminobutyrate were only little affected.

Effect of neonatal thyroidectomy on the manifestation of metabolic compartmentation. This was followed by determining the specific radioactivity of the amino acids associated with the tricarboxylic acid cycle relative to that of glutamate. The values obtained for glutamine indicated that thyroid deficiency resulted in a marked retardation in the development of metabolic compartmentation (Fig. 3a).

The aspartate/glutamate specific-radioactivity ratio changed little in the normal brain during development, and the value was not significantly affected by neonatal thyroidectomy (Fig. 3b). In contrast, the γ -aminobutyrate/glutamate specific-radioactivity ratio increased with age in the brain of normal rats (Fig. 3c). Neonatal thyroidectomy resulted in a significant decrease in the specific radioactivity of γ -aminobutyrate relative to that of glutamate. Thus the age curve for γ -aminobutyrate both under normal conditions and in thyroid deficiency followed that for glutamine.

Effect of thyroid state on the concentration of amino acids. In spite of the marked effects of the thyroid state on the development of metabolic compartmentation in brain, the maturational changes in the concentration of amino acids were relatively small. Treatment with tri-iodothyronine had no significant effect on the concentrations of amino acids

Table 4. *Effect of neonatal thyroidectomy on the metabolism of free leucine in the brain during development*

Age (days)	Experimental treatment		10 ⁻³ × Sp. radioactivity of leucine (d.p.m./μmol)	10 ³ × Sp. radioactivity of constituent relative to that of leucine				
				Protein	Amino acids*	Glutamate	Aspartate	γ-Amino-butyrate
18	Mean	T _x	308	0.97	1.83	1.91	0.97	2.74
		N	344	1.36	1.43	1.38	0.94	2.47
	Percentage change:							
		T _x /N	90	71	130	138	103	111
25	Mean	T _x	335	0.79	2.36	2.47	1.53	2.90
		N	260	0.95	1.35	1.20	0.81	2.66
	Percentage change:							
		T _x /N	129	83	175	206	189	109
	25/18	T _x	109	81	129	129	158	106
		N	76	70	94	93	86	108
S.E.			11	0.05	0.07	0.09	0.07	0.25
Significance:								
Normal (25/18)			<0.01	<0.01	N.S.	N.S.	N.S.	N.S.
T _x (25/18)			N.S.	<0.05	<0.01	<0.01	<0.01	N.S.
18 day (T _x /N)			N.S.	<0.01	<0.01	<0.01	N.S.	N.S.
25 day (T _x /N)			<0.01	<0.05	<0.01	<0.01	<0.01	N.S.

* The specific radioactivity of 'cycle amino acids' was calculated in terms of the content of ¹⁴C (d.p.m.) and the sum of glutamate, glutamine, aspartate and γ-aminobutyrate (μmol).

(Table 5a). In thyroid deficiency the concentration of glutamate was decreased by about 13 and 10% at the age of 12 and 18 days respectively, but it was comparable with the normal values at 25 days (Table 5b). The concentration of alanine was decreased by 20% at 18 days, and that of aspartate by 25% at 25 days after birth. The concentrations of the other amino acids studied, such as glutamine, γ-aminobutyrate and leucine, were not significantly different from controls.

DISCUSSION

Age-dependent changes in the labelling of brain constituents after administration of [U-¹⁴C]leucine. The changes during development in the labelling of brain constituents (Fig. 1) are related to a number of different factors. It has been suggested that the uptake of blood-borne metabolites such as leucine depends mainly on the metabolic requirements of the tissues (Dobbing, 1961). During the

early postnatal period the brain is growing more rapidly than the body as a whole; but during the later period of rapid body growth, organs other than brain will remove [¹⁴C]leucine more rapidly from the blood. Thus the labelling of brain constituents depends, not only on brain metabolism, but also on the availability of [¹⁴C]leucine in the blood. This agrees with the age-curves obtained for the specific radioactivity of free leucine and the ¹⁴C content of the brain (Fig. 1).

The specific radioactivity of free leucine in the brain is also related to other factors, such as the transport of leucine within the brain, the size of the endogenous pool and the rate of the overall metabolism of leucine. It seems that some at least of these factors must change during the period of cerebral maturation. The fall in concentration of free leucine in the brain may contribute to the rise in specific radioactivity of leucine observed between 9 and 12 days of age, but this would not explain the increase in the total ¹⁴C content of the brain. In

Table 5. *Effects of thyroid state on the concentrations of cerebral amino acids during development*

		Concentration ($\mu\text{mol/g}$ wet wt.)					
Age (days)	Experimental treatment	Glutamate	Glutamine	Aspartate	γ -Amino-butyrate	Alanine	Leucine
9	Tri-iodothyronine	5.84	4.43	1.71	1.29	1.15	0.130
	Normal	6.13	2.83	1.48	1.30	1.19	0.156
12	Tri-iodothyronine	7.38	4.23	1.94	1.56	0.83	0.112
	Normal	7.11	4.06	1.89	1.42	0.87	0.099
15	Tri-iodothyronine	8.81	4.35	2.61	1.50	0.64	0.056
	Normal	7.79	3.65	2.03	1.56	0.67	0.068
21	Tri-iodothyronine	10.12	3.75	2.94	1.61	0.65	0.063
	Normal	10.46	4.06	3.09	1.66	0.66	0.070
S.E.		0.27	0.26	0.15	0.09	0.10	0.012
Significance:							
'Age'		<0.01	N.S.	<0.01	<0.05	<0.01	<0.01
'Treatment'		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

		Concentration ($\mu\text{mol/g}$ wet wt.)					
Age (days)	Experimental treatment	Glutamate	Glutamine	Aspartate	γ -Amino-butyrate	Alanine	Leucine
12	T_x	6.58	3.54	1.90	—	—	—
	Normal	7.53	3.19	2.08	—	—	—
18	T_x	8.75	3.83	2.46	1.73	0.70	0.119
	Normal	9.60	2.93	2.72	1.75	0.85	0.086
25	T_x	10.69	3.22	2.38	1.79	0.55	0.085
	Normal	10.96	3.62	3.17	1.73	0.59	0.079
S.E.		0.27	0.35	0.10	0.08	0.03	0.013
Significance:							
'Age'		<0.01	N.S.	<0.01	N.S.	<0.01	N.S.
'Treatment'		<0.01	N.S.	<0.01	N.S.	<0.05	N.S.

quantitative terms the main pathway of leucine metabolism in the brain is incorporation into protein (Tables 1 and 2), and Fig. 1 shows that the increase in the ^{14}C combined in protein accounts for most of the increased ^{14}C content in the brain. It has been observed in this laboratory that the rate of [^{14}C]leucine incorporation into cerebral proteins changes characteristically during maturation (Patel & Balázs, 1970, and R. Balázs, S. Kovács, W. A. Cocks & A. J. Patel, unpublished work). The rate increases rapidly between 9 and 12 days after birth and decreases after about 21 days. Thus the period of maximal rate of protein synthesis coincides with that when the morphological and biochemical maturation of brain tissue is most extensive (Eayrs & Goodhead, 1959; Aghajanian & Bloom, 1967; Cocks *et al.* 1970). The rate of leucine oxidation also increases significantly from 9 to 12 days of age and the rate remains high later (Fig. 1b).

The operation of a barrier system in the passage of metabolites between blood and brain, which is

also subject to developmental changes, has previously been considered (cf. Lajtha, 1968). The present observations can be accounted for to a large extent on other assumptions (cf. above). Ford & Rhines (1969), who studied the incorporation of [^3H]lysine into brain proteins, have reached similar conclusions. It has also been shown that, although there may be differences at different ages in the leucine content of the brain after the injection of relatively large amounts of leucine (Lajtha & Toth, 1961), both in the immature and in the adult animal there is a rapid equilibration of [^{14}C]leucine between blood and brain (Lajtha, 1959; Roberts & Morelos, 1965) and the uptake of [^{14}C]leucine is relatively uniform in the different regions (Kandera, Levi & Lajtha, 1968; Berl & Frigyesi, 1969).

Effect of thyroid state. (a) Neonatal thyroidectomy. It was observed that in comparison with the normal animal, less leucine carbon was converted into cerebral proteins and lipids, and more into the amino acids associated with the tricarboxylic acid cycle (Table 2).

The rate of incorporation in protein was decreased by 20–30% in thyroid deficiency (Table 4; cf. Geel, Valcana & Timiras, 1967; Balázs, Cocks, Eayrs & Kovács, 1970a). Since the supply of blood-borne [^{14}C]leucine is presumably maintained, this could lead to a 10–15% increase in free [^{14}C]leucine in the brain. The higher concentration of [^{14}C]leucine may be expected to result in a rise, in comparison with normal animals, in the ^{14}C combined in metabolites such as amino acids associated with the path of leucine breakdown. Thus an increase in the ^{14}C content of free amino acids in the brain could occur in thyroid-deficient rats, without an increase in the rate of oxidation of leucine.

The increase in the conversion of leucine carbon affected glutamate and aspartate, but not glutamine or γ -aminobutyrate (Tables 3 and 4). This observation helped the effects of thyroidectomy to be better understood, since it indicated the involvement of the metabolic compartmentation in the brain. The metabolic heterogeneity of brain tissue can be described on a functional basis in terms of a 'large' and a 'small' compartment (reviews by Berl & Clarke, 1969; Van den Berg, 1970). Each compartment results from the summation of a number of units of more or less similar metabolic pattern (Balázs, Machiyama, Hammond, Julian & Richter, 1970c). The 'small' compartment appears to be associated with a relatively small glutamate pool, but it is the site of the major part of [^{14}C]glutamine formation as a result of utilization of labelled substrates that elicit the signs of metabolic compartmentation. The 'large' compartment seems to contain the major part of glutamate (for more detailed characterization of the functional compartments, see Patel & Balázs, 1970). Circumstantial evidence has been presented that glial tissue is associated mainly with the 'small' compartment, whereas the 'large' compartment consists primarily of neuronal structures (Balázs *et al.* 1970c; Machiyama, Balázs, Hammond, Julian & Richter, 1970a; Machiyama, Balázs & Mérei, 1970b; Patel & Balázs, 1970; Cocks *et al.* 1970).

There is evidence that the rate of incorporation of labelled amino acids into protein is higher in the perikarya of nerve cells than in the glial cells (Oehlert, Schultze & Maurer, 1958; Richter, Gaitonde & Cohn, 1960; Ford, Rhines, Hartstein & Rhodes, 1965; Blomstrand & Hamberger, 1969; Rose, 1969). Thus the decrease in the rate of protein synthesis in thyroid deficiency implies a decreased incorporation of free leucine into protein with a corresponding rise in the free [^{14}C]leucine, which would be relatively greater in the neurons than in the glia. Since the 'large' glutamate pool is situated in the neuron, the preferential rise in free [^{14}C]leucine would be expected to lead to an increased labelling of glutamate. The incorporation

of ^{14}C into glutamine was only little affected in thyroid deficiency, and this is consistent with the assumption that [^{14}C]glutamine formation is associated primarily with the glutamate pool in the 'small' compartment, i.e. in the glial cells.

The ^{14}C content of aspartate increased in parallel with that of glutamate in the brain of thyroid-deficient rats (Tables 3 and 4; Fig. 3b). The results indicate therefore that most of the free aspartate, like the free glutamate, is associated with the 'large' metabolic compartment.

The steady-state concentrations of brain constituents are only slightly affected in thyroid deficiency (Balázs, 1970). It has been reported by Ramírez de Guglielmo & Gómez (1966), Mussini, Marcucci & Garattini (1967) and Marcucci & Airolidi (1969) that there is a transient change in the content of some amino acids after neonatal thyroidectomy. These observations were confirmed in the present work, which also showed that the effect is small. In the case of glutamate it had completely disappeared at 25 days of age (Table 5); at that age the glutamine/glutamate specific-radioactivity ratio was decreased by 47% compared with the normal (Fig. 3a).

(b) Treatment with thyroid hormone. The effects of thyroid treatment on the development of metabolic compartmentation were broadly the opposite of those of thyroidectomy; the age-dependent increase in the glutamine/glutamate specific-radioactivity ratio was advanced in the brain of animals treated with thyroid hormone (Fig. 3a). However, in contrast with thyroid deficiency, the normal increase during development in the γ -aminobutyrate/glutamate specific-radioactivity ratio remained apparently unchanged (Fig. 3c); this may only be apparent, since the effects of tri-iodothyronine treatment on leucine metabolism compared with those of thyroid deficiency were quantitatively small (compare Table 1 with Table 2). The advancement of metabolic compartmentation by treatment with tri-iodothyronine was not associated with a significant effect on the incorporation of [^{14}C]leucine into proteins in the whole cerebrum (Table 1, and Balázs *et al.* 1970a). The results suggested therefore that the treatment affected mainly the reactions associated with the oxidation of [^{14}C]leucine in the developing metabolic compartments of the tissue.

Thyroid treatment also advances the maturation of cerebral energy metabolism. Cocks *et al.* (1970) have related the increased rate of conversion of glucose carbon into amino acids to the development of specific neuronal structures during maturation. Patel & Balázs (1970) have reported evidence that the manifestation of metabolic compartmentation depends on changes in glial-neuronal relations involving an expansion of the 'large' (neuronal)

compartment relative to the 'small' (glial) compartment. The effects of the thyroid status on the morphological and biochemical maturation of the brain are consistent with these views. Thyroid deficiency leads to a decrease in the formation of dendrites and nerve terminals (Eayrs, 1968; Balázs *et al.* 1968; Cragg, 1970), whereas treatment with thyroid hormone results in an acceleration of the formation of dendritic spines on cortical neurons (Schapiro, 1968). An acceleration of neuronal maturation is also indicated by the effects of thyroid treatment on innately organized behaviour and on the electrophysiological manifestations of neuronal activity (Eayrs, 1968; Schapiro, 1968).

Much less information is available on the effects of the thyroid state on the development of glial cells. Myelination may be taken as an index of the differentiation of the oligodendroglia. Myelination is quantitatively decreased in the brain of thyroid-deficient rats, but this could relate to a decrease in axonal density (Balázs *et al.* 1969). Myelination is not markedly affected in the brain of tri-iodothyronine-treated rats (Balázs, 1970). Tri-iodothyronine treatment leads, however, to a considerable decrease in the postnatal cell formation, which would affect predominantly the glial cells in the rat cerebrum (Balázs, Kovács, Cocks, Johnson & Eayrs, 1970b). It is therefore notable that the development of metabolic compartmentation was not adversely affected by treatment with tri-iodothyronine, as would be expected if this phenomenon were primarily related to the metabolism of glial cells. The effects of thyroid state on brain maturation thus corroborate the hypothesis that metabolic compartmentation depends on glial-neuronal relations; they also support the allocation of the 'large' metabolic compartment to neuronal structures and the association of the 'small' compartment with glial cells (Balázs *et al.* 1970c). The present results indicate further that the 'large' metabolic compartment apparently contains the sites of active protein synthesis in the neuronal cell bodies. Although thyroid state has a marked effect both on the conversion of glucose carbon into amino acids (Cocks *et al.* 1970) and on the manifestation of metabolic compartmentation (Fig. 3a), it influenced only slightly the development of the amino acid profile in the brain (Table 5). It seems, therefore, that the steady-state concentration of a tissue constituent is not necessarily a sensitive marker for metabolic changes in a tissue, although the particular constituent studied may show a well-defined maturational change. On the other hand, markers that reflect the dynamic properties of the tissue, such as glucose metabolism and metabolic compartmentation, provide sensitive indices of brain development.

Relationship between γ -aminobutyrate and glut-

amine. A special relationship between γ -aminobutyrate and glutamine was suggested by the labelling pattern of amino acids during normal development and also in thyroid deficiency (Figs. 2 and 3). Evidence was previously presented that a significant part of γ -aminobutyrate formation takes place in the metabolic compartment (nerve terminals), which is different from that in which the utilization of γ -aminobutyrate and the labelling of glutamine by other 'compartmentation substrates' occur ('small' or 'glial' compartment, Balázs *et al.* 1970c; Machiyama *et al.* 1970a). Berl, Lajtha & Waelsch (1961) have shown that exogenous glutamine is converted into γ -aminobutyrate faster than externally added glutamate. There is also some evidence for the view that γ -aminobutyrate may have a transmitter role in the central nervous system (Curtis & Watkins, 1965; Krnjević & Schwartz, 1967); thus the loss of carbon units at the site of γ -aminobutyrate release must be compensated to ensure the unimpeded operation of the tricarboxylic acid cycle. An input of glutamine formed in metabolic compartments other than those associated with the synthesis and release of γ -aminobutyrate would serve to maintain the homeostasis of the system. These assumptions are consistent with the present observations, which showed that the labelling of γ -aminobutyrate followed that of glutamine both during normal development and in thyroid deficiency (Fig. 3). C. J. Van den Berg & D. Garfinkel (personal communication) have independently observed that a model in which communication between the 'large' and the 'small' compartment is mediated through glutamine and γ -aminobutyrate respectively allows for a satisfactory simulation of the observed labelling of amino acids by different radioactive substrates. The metabolic relationship observed in the present work between labelled glutamine and γ -aminobutyrate, which are formed predominantly in the 'glial' and 'neuronal' compartments respectively, support previous suggestions of a metabolic interconnexion between glial cells and at least certain structures of the neurons (reviews by Hydén, 1960; Galambos, 1964).

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