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INCORPORATION OF ^{14}C FROM GLUCOSE INTO α-KETO ACIDS AND AMINO ACIDS IN RAT BRAIN AND LIVER *IN VIVO*

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Abstract—The incorporation *in vivo* of ^{14}C from glucose into the α-keto acids and amino acids of rat brain and liver was studied over the first 5–30 min after injection of uniformly labelled ^{14}C-glucose. The brain intermediates were all more highly labelled than those from the liver. During the first 5 min, the brain alanine, pyruvate and α-oxoglutarate were significantly more highly labelled than the other brain amino acids (glutamate, glutamine, aspartate and γ-aminobutyrate). By 30 min the radioactivity of the brain keto acids and aspartate was significantly lower than the radioactivity of the other brain amino acids. No significant difference in the incorporation of ^{14}C into brain glutamate, glutamine or γ-aminobutyrate was observed at any of the time intervals studied. The results are discussed in terms of the "metabolic compartments" which have previously been postulated for the free amino acid pools of brain.

INTRODUCTION

PREVIOUS investigations into the utilization of ^{14}C-glucose by mammalian tissues[1–10] have demonstrated the high rate of incorporation of ^{14}C into brain amino acids *in vivo* and *in vitro*. Emphasis has been placed on the relatively high radioactivity of the free glutamate pool,[2, 3, 9] and the differences observed in the ^{14}C incorporation into different amino acids from ^{14}C-glucose have been used as arguments in favour of the existance of metabolic compartments within the brain amino acids pools.[6, 7, 10] However, the results which have so far been presented are not conclusive as little statistical analysis of the results has been made. Statistical treatment of such results would appear to be essential in the interpretation of *in vivo* isotopic labelling studies in view of the inevitably large variations between individual members of identically-treated groups of experimental animals.[13]

The brain amino acids which have been studied extensively are those (glutamate, glutamine, aspartate and γ-aminobutyrate) which are related, directly or indirectly, to intermediates of the tricarboxylic acid cycle. Little attention[1, 2, 6] has been paid to alanine, which by virtue of its relation via transamination reactions to pyruvate, is of interest in the pathways of utilization of glucose carbon. Alanine poses technical problems in isolation and purification as it occurs in the brain free amino acid pool in very small amounts[14] relative to the other amino acids of interest. Similarly, the α-keto acids of relevance to the incorporation of glucose carbon into amino acids (pyruvate, oxaloacetate and α-oxoglutarate), which occur in trace amounts[15] in the brain, have received little attention.

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This communication reports a statistical analysis of the labelling of glutamate, glutamine, aspartate, γ-aminobutyrate, alanine, pyruvate and α-oxoglutarate in rat brain and liver at various times after the subcutaneous injection of uniformly-labelled ^{14}C-glucose. A preliminary report on the labelling of the α-keto acids has appeared.[13]

EXPERIMENTAL

Treatment of animals

Male rats (100–120 g) of the Wistar Albino Glaxo strain were injected subcutaneously with carrier-free U-^{14}C-glucose (15μc/100 g body weight). At various times after injection, the animals were killed by decapitation, the blood was immediately collected in heparinized beakers, and deproteinized by pipetting a measured volume (usually 1 ml) into ice-cold 6% (w/v) perchloric acid. Also immediately after death the brain and liver were rapidly excised, blotted and frozen by immersion in liquid N_2. The time from decapitation to complete freezing of the tissues was usually within 1 min and never exceeded 1·5 min. The tissues were weighed, dropped while still frozen into cold 6% (w/v) perchloric acid (6 ml/g), dispersed in a Teflon-pestle homogenizer (Type B, A. H. Thomas Co., Philadelphia), and the dispersion was centrifuged at 1500 × *g* for 15 min. The precipitate was washed once with cold 5% (w/v) perchloric acid and the supernatant plus washings were combined. Two-thirds of this extract was taken for immediate isolation of α-keto acids as these intermediates tend to deteriorate on standing.[15] The remainder of the extract was stored at −20° for the subsequent isolation of amino acids. The isolation of metabolites is summarized in the flow-sheet (Fig. 1).

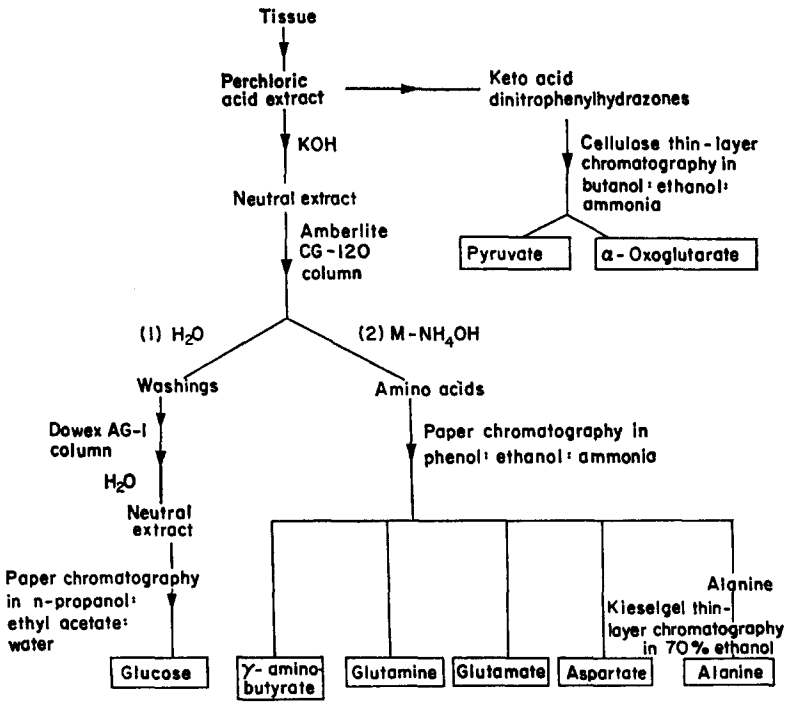

FIG. 1. Summary of isolation of tissue metabolites.

Isolation of α-keto acids

The tissue perchloric acid extracts were immediately reacted with one-tenth the volume of 2 : 4-dinitrophenylhydrazine (0·2% in 2M-HCl) for 30 min. The keto-acid dinitrophenylhydrazones were extracted and purified as described previously[13] and separated (Fig. 1) by cellulose thin-layer chromatography[16] in the dark in butan-1-ol: ethanol: 0·5M–NH_4OH (7: 1: 2 by vol). The dinitrophenylhydrazone derivatives of pyruvate and α-oxoglutarate were scraped off the plate into micro-test tubes and eluted into cold 10% (w/v) Na_2CO_3 (0·5 ml) by "buzzing" and rapid centrifugation in a Servall SSI. The extracts were acidified with conc. HCl at 0° by buzzing (Vortex shaker) in the presence of ethyl acetate (0·6 ml). The ethyl acetate extract was removed, dried over anhydrous Na_2SO_4 and estimated directly at 360 mμ.[13] In all the tissue extracts, in which storage time under acid conditions[16] was kept to a minimum (the extractions and estimations were all performed within the same day under controlled conditions of light and temperature) no separation of *syn-anti* isomers[16] during chromatography was observed. Recoveries of added keto acids to the tissue extracts ranged from 96 to 102 per cent.

The same ethyl acetate extracts, in which the keto acid dinitrophenylhydrazones had been estimated spectrophotometrically were counted in a Packard "Tricarb" model 314 EX liquid scintillation counter at −10° in the scintillator described by Patrick,[17] except that it was found necessary to include dioxane (10 per cent by vol). The absolute efficiency was 70 per cent; no quench correction was found to be necessary for dinitrophenyhydrazones up to 15 μg in 10 ml scintillator. Results were reduced to a calculated 65 per cent efficiency for comparison with glucose and amino acids.

Isolation of amino acids

The remaining one-third of the original tissue perchloric acid extract was neutralized (pH 7·0–7·5) with cold 10 per cent (w/v) KOH, held at 3° overnight and centrifuged (1000 *g*, 10 min). The $KClO_4$ precipitate was washed once with ice-cold water. The combined neutral extract and washings were passed over a column (5 × 1 cm) of Amberlite CG-120 (H^+ form) and the column was washed with 8 column volumes of water. The amino acids were eluted from the column using M-NH_4OH (5 column volumes).

Individual amino acids were separated from the lyophilised column eluate by descending chromatography on Whatman No. 3. MM paper in a phenol: ethanol: ammonia solvent made up[18] from phenol (60 g), ethanol (20 ml), water (20 ml), and 0·880 NH_4OH (0·5 ml). The solvent was allowed to run over the edge of the paper; the extent of development was followed by a visible dye marker (Shandon Unikit No. 202), the orange component of which had an R*f* value of 0·70, slightly faster than the leading amino acid (γ-aminobutyrate) (Table 1).

The developed chromatograms were air dried, the phenol was removed by washing with acetone and the positions of the amino acids in the tissue extracts were detected by means of marker strips of a synthetic amino acid mixture run on the same chromatogram. The areas corresponding to individual amino acids were cut out and eluted chromatographically with 0·1 M-HCl. Two-dimensional chromatography of the eluted amino acids in (i) ethanol: water: 0·880 ammonia (80: 10: 10 by vol) and (ii)

butan-1-ol: acetic acid: water (60: 15: 25 by vol) confirmed the purity of the isolated amino acids. Recoveries of amino acids were 75–90 per cent.

The amino acids were estimated by the method of Yemm and Cocking[19] as modified by Matheson and Pattrie[20] to reduce the colour yield of peptides eluted from the paper. Paper blanks, cut from the chromatograms, were also eluted and estimated. For each amino acid except alanine the non-amino acid ninhydrin-reacting material contributed less than 10 per cent of the colour yield. However, with alanine, which occurs in brain in small amounts, the paper blank value led to a potential error of ±55 per cent. Pre-washing the paper[18] was not effective in lowering the paper blank. Accordingly the alanine was further purified by thin-layer chromatography on Kieselgel "H" in 70 per cent (v/v) ethanol. The location of the alanine was deduced from marker strips. After elution of the alanine in 0·01 M-HCl, the plates were sprayed with ninhydrin—all of the alanine had been removed from the plate and the paper contaminant was revealed at the origin. The recovery of alanine during thin layer chromatography was 85–90 per cent. Errors in estimation of the purified alanine were estimated to be less than 5 per cent, compared with over 50 per cent before thin-layer chromatography.

TABLE 1. PAPER CHROMATOGRAPHY OF AMINO ACIDS IN PHENOL:ETHANOL:NH_4OH

Amino acid	R_f	RGABA
γ-Aminobutyrate (GABA)	0·66	1·00
Alanine	0·50	0·75
Glutamine	0·41	0·62
Glycine	0·32	0·48
Serine	0·27	0·41
Glutamate	0·16	0·24
Aspartate	0·10	0·15
Dye marker (Unikit No. 202)	0·70	

Radioactivity of the purified amino acids was determined in the Packard counter in the scintillator described by Bruno and Christian.[21] Absolute efficiency was 65 per cent.

Glucose

The water washings from the Amberlite CG 120 columns were passed through columns (6 × 1 cm) of Dowex AG1 (Acetate form) to remove anions, and the columns were washed with 8 column volumes of water. The washings were lyophilised, the residue was dissolved in the minimum volume of water and the glucose was isolated by paper chromatography,[22] estimated using glucose oxidase[23] and the radioactivity was determined as described for the amino acids.

Statistical Analysis

The Student *t*-test was used throughout for estimating the significance of differences between means, after using the "*F*" (Variance Ratio) test to determine compatibility of variances. Where variances were incompatible, a modified *t*-test[24] was used.

Reagents

All reagents were of analytical purity. Amberlite CG 120 (×8, 200–400 mesh) was obtained from the Mallinckrodt Chemical Works, N.Y., U.S.A. and Dowex-AG-1 (×8, 200–400 mesh) from Biorad Laboratories, Richmond, U.S.A. Cellulose MN 300 powder was from Machery, Nagel and Co., Duren, Germany and Kieselgel "H" from E. Merck A.G. Darmstadt, Germany. U-^{14}C-glucose (47·4 μc/mg) was provided by the Radiochemical Centre, Amersham, U.K.

RESULTS

Rates of incorporation of ^{14}C from glucose into α-keto acids and amino acids of rat brain and liver

The specific activities of the isolated intermediates (α-keto acids and amino acids) increased rapidly with time, particularly in the brain (Table 2). The high incorporation of ^{14}C from glucose into the brain amino acids in comparison with the liver amino acids confirms previous reports.[1–9] In the present study the same pattern is shown for the keto acids—the brain pyruvate and α-oxoglutarate were 3–4 times more highly labelled than the liver keto acids 30 min after the injection of ^{14}C-glucose.

TABLE 2. SPECIFIC ACTIVITIES OF α-KETO ACIDS AND AMINO ACIDS ISOLATED FROM RAT BRAIN AND LIVER AFTER SUBCUTANEOUS INJECTION OF ^{14}C-GLUCOSE

Tissue	Metabolite	Time after ^{14}C-glucose injection (min)		
		5	10	30
Brain	Pyruvate	1230 ± 200	2200 ± 190	7740 ± 750
	α-Oxoglutarate	1500 ± 190	2310 ± 490	7250 ± 990
	Alanine	1220 ± 180	1630 ± 340	13,870 ± 250
	Aspartate	410 ± 30	620 ± 100	8680 ± 540
	Glutamate	500 ± 30	950 ± 130	11,710 ± 440
	Glutamine	480 ± 20	910 ± 170	11,680 ± 1310
	γ-Aminobutyrate	780 ± 70	1000 ± 170	10,460 ± 450
Liver	Pyruvate	370 ± 60	730 ± 140	2320 ± 240
	α-Oxoglutarate	180 ± 8	520 ± 110	2010 ± 200
	Alanine	410 ± 80	1070 ± 190	5610 ± 100
	Aspartate	130 ± 5	300 ± 50	1560 ± 280
	Glutamate	100 ± 30	210 ± 30	1910 ± 190
	Glutamine	260 ± 40	570 ± 60	6240 ± 160
Blood	Glucose	8120 ± 1720	18,360 ± 4110	51,910 ± 5860

Specific activity: counts/min/μmole ± S.E. Each value is the mean of the results from 4 animals.

The ^{14}C incorporation into the brain keto acids and into alanine was clearly higher than the incorporation into the other brain amino acids in the first few minutes after glucose injection. However, 30 min after injection, the level of radioactivity of the keto acids was below those of all of the amino acids; alanine at 30 min was little different from glutamate. No differences were apparent in the incorporation of label into glutamate, glutamine or γ-aminobutyrate.

The rates calculated for the incorporation of ^{14}C over the various time intervals are shown in Table 3. In the brain, the rate of incorporation into the keto acids (pyruvate and α-oxoglutarate) was relatively constant over the period studied, in contrast to all the amino acids, for which the rates increased considerably after 10 min. The contrast

in the rates of labelling between keto acids and amino acids was less marked in the liver.

For statistical analysis of the extent of labelling in the isolated metabolites from the brain, the relative specific activities (counts/min/μmole of metabolite vs counts/min/μmole blood glucose) were calculated since expression of the results on this basis lessens the scatter due to variations, within the same experimental group, in the radioactivity of the circulating glucose (Table 1). These appear in Table 4.

TABLE 3. RATES OF INCORPORATION OF ^{14}C INTO α-KETO ACIDS AND AMINO ACIDS DURING VARIOUS TIME INTERVALS AFTER INJECTION OF ^{14}C-GLUCOSE

Tissue	Metabolite	Time period (min)		
		0–5	5–10	10–30
Brain	Pyruvate	245	200	275
	α-Oxoglutarate	300	160	245
	Alanine	240	80	610
	Aspartate	80	40	400
	Glutamate	100	90	540
	Glutamine	100	90	540
	γ-Aminobutyrate	160	40	470
Liver	Pyruvate	75	70	80
	α-Oxoglutarate	35	65	75
	Alanine	80	130	230
	Aspartate	30	30	60
	Glutamate	20	20	80
	Glutamine	50	60	280
Blood	Glucose	1650	2000	1700

The rate of incorporation is expressed as the mean rate over the time elapsed: counts/min/μmole/min. Each value is the mean of the results from four animals.

TABLE 4. RELATIVE SPECIFIC ACTIVITIES OF BRAIN METABOLITES AT VARIOUS TIMES AFTER ^{14}C-GLUCOSE INJECTION

Metabolite	Time (min) after ^{14}C-glucose injection		
	5	10	30
Pyruvate	15·5 ± 1·3*	14·2 ± 2·3*	13·8 ± 1·0*
α-Oxoglutarate	16·9 ± 2·2*	13·3 ± 3·4*	13·0 ± 0·9*
Alanine	17·9 ± 3·0*	10·0 ± 2·3	27·6 ± 3·0
Aspartate	5·7 ± 1 1	3·6 ± 0·5	16·9 ± 0·4*
Glutamate	7·0 ± 1·4	5·5 ± 0·8	22·5 ± 1·5
Glutamine	6·8 ± 1·6	5·0 ± 0·3	22·1 ± 3·8
γ-Aminobutyrate	11·4 ± 3·3	5·8 ± 1·0	20·8 ± 1·7

* Significantly different from glutamate at the same time ($P < 0·05$).
Relative specific activity: Counts/min/μmole metabolite vs. Counts/min/μmole blood glucose × 10^2 ± S. E. Each value is the mean of the results from four animals.

The brain keto acids were significantly more highly labelled than glutamate, aspartate, glutamine or γ-aminobutyrate 5 and 10 min after the injection of ^{14}C-glucose, and were significantly lower than glutamate, glutamine, γ-aminobutyrate or alanine at 30 min. A higher radioactivity at 5 min was also observed in alanine and

was significantly higher than that of glutamate. At 10 or 30 min no difference between alanine and glutamate was observed. The only other difference was that aspartate was significantly lower in radioactivity than the other amino acids at 30 min. There was no significant difference between glutamate, glutamine or γ-aminobutyrate at any of the time intervals studied.

DISCUSSION

The constancy of the rate of labelling of the keto acids in contrast to the increased rate of labelling of the amino acids, all of which could be expected to become labelled by isotopic exchange via transamination reactions directly or indirectly, indicates that a proportion of the glucose carbon is continuously and cumulatively channelled into the amino acids, i.e. the transamination reactions *in vivo* would appear to be in favour of amino acid formation rather than the reverse. Such a retention of radioactivity in the amino acid pools after isotopic exchange between α-oxo acids and the corresponding amino acids has been suggested by Haslam and Krebs.[25] Such evidence lends further support to the view[13, 25] that the high rate of labelling of glutamate from ^{14}C-glucose in mammalian brain *in vivo* is more likely to be due to active glycolysis and isotopic exchange between α-keto acids and amino acids, than to the special role which has been postulated[4] for glutamate in cerebral glucose metabolism.

The lower radioactivity of aspartate, 30 min after injection, in confirmation of previous reports,[7, 26] may be due to a dilution of ^{14}C in the tricarboxylic acid cycle subsequent to α-oxoglutarate. It is unlikely to be due solely to direct CO_2-fixation which is considered[27, 28] to contribute less than 10 per cent of the carbon of brain aspartate.

No significant differences were observed in the present study in the labelling of glutamate, glutamine or γ-aminobutyric acid at any time up to 30 min after the injection of ^{14}C-glucose. Such results provide no evidence for the existence of metabolic compartments as suggested from previous studies. Waelsh and his co-workers[11] reported that from 2 to 60 min after administration of ^{14}C-glutamate via the cerebrospinal fluid the specific activity of the brain glutamine was considerably higher than that of the brain glutamate. Similar observations were made[12] for the α-NH_2 group of glutamine, compared with glutamate, after intracarotid injection of $^{15}NH_4OH$. It was postulated that the glutamine was formed from an "active" pool of glutamate and that during the subsequent purification of the glutamate, the "active" pool was diluted by an "inactive" pool. It was emphasized that this effect could only be detected in a short-term experiment, before equilibration of the pools occurred.

Other studies[6, 7] on the labelling of glutamate and glutamine in brain *in vivo* from ^{14}C-glucose have produced findings similar to those reported here—that no difference in labelling between glutamate and glutamine occurred. Thus the high specific activity of glutamine, relative to glutamate, reported[11, 12] may be a reflection of the precursor used or of the different route of administration.

The existence of "active" and "inactive" glutamate pools have also been postulated from differences claimed[6, 7, 10] between the specific activities of γ-aminobutyrate and glutamate isolated from brain after *in vivo* ^{14}C-glucose administration. The observed differences were small; either no statistical evaluation was presented, or if it was,[6]

no significant differences to support the claims were found. It seems doubtful, therefore, if the observed differences were significant, especially in view of the unavoidable variations in the results from studies of this type.

The results of a careful statistical analysis of the extent of incorporation of ^{14}C from glucose into glutamate, glutamine and γ-aminobutyrate presented here provide no evidence for "compartmentation" of these amino acids in brain. If "active" and "inactive" pools of glutamate do occur, it is unlikely that definitive conclusions can be drawn from studies on the ^{14}C incorporation from the circulating glucose *in vivo* into intermediates isolated from whole tissue.

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