Entry of Water, Metabolic Substrates and Extracellular Space Markers into Various Structures of Mouse Brain in vivo[1]

It is well known that after systemic injection of radioactive water into living animals it exchanges rapidly with brain water, equilibration between brain and plasma water being attained within a few minutes[2,3]. On the other hand, intra-ventricularly injected or ventriculo-cisternally perfused sucrose and inulin do not enter brain water rapidly and upon equilibration these substances are confined to the 'extra-cellular space'[4]. Since glucose is the primary energy substrate of brain[5,6] and since fructose and pyruvate are readily converted into glucose peripherally (e.g., in liver), carbon atoms of these substances enter brain rapidly in vivo[7–15]. Although a number of studies have been undertaken to examine the incorporation of these various substances into brain, or brain structures, the 'simultaneous' incorporation of energy substrates and space markers (as reference substances) has not been determined in vivo. In an effort to devise more sensitive methods for studying the effects of environmental changes and psychoactive agents on brain metabolism it was considered pertinent to study the simultaneous incorporation of carbon atoms of metabolic substrates and of labelled atoms of substances which mark brain spaces into various structures of the brains of living mice.

Adult, male, C-57B mice, weighing 22–30 g (Indianapolis Laboratory Supply Co.), were kept in groups of 12 for 3 days before experiments. All animals were fasted 12–24 h prior to experiments which were conducted during the autumn months. All injections were made s.c. (neck region) 30 min before sacrifice of animals. Solutions of [U-^{14}C] D-glucose 206 mCi/mmole), [U-^{14}C] D-fructose (121 mCi/mmole), [1-^{14}C] pyruvate (5.9 mCi/mmole), [U-^{14}C] D-sucrose (505 mCi/mmole), [^{3}H] inulin (0.38 mCi/mg) and [^{3}H]OH (25 mCi/g) were made up in 0.154 *M* NaCl. (All radioactive substances were purchased from New England Nuclear Corp. except for [^{3}H] inulin which was from Schwarz BioResearch, Inc.). Injected doses of [^{3}H]OH and [^{3}H] inulin were 0.25 μCi/g, those of [U-^{14}C] D-glucose and [U-^{14}C] D-sucrose were 0.1 μCi/g and those of [U-^{14}C] D-fructose and [1-^{14}C] pyruvate were 0.05 μCi/g, mouse. Note that equilibration of extracellular space markers was not expected to occur in 30 min; these were used as 'control' substances in this work.

After decapitation of mice the cerebral cortex, cerebellum, olfactory bulbs, hippocampus, diencephalon (i.e., thalamus, hypothalamus and other structures lying close to these), spinal cord and colliculi were carefully dissected out on a chilled surface, weighed, and immersed in 2.0 ml of ice-cold 80% ethyl alcohol solution (v/v). All samples were homogenized and ethanolic extracts were prepared[16]. Radioactivity was determined in aliquots of homogenates and extracts. (Since values for homogenates and extracts were quite similar, only values for homogenates are presented herein). The 'counting cocktail' consisted of 240 g naphthalene, 1 l toluene, 1 l, 1,4-dioxane, 1 l ethyl alcohol (abs.), 0.3 g dimethyl-POPOP and 15 g PPO[17]. Correlation curves were constructed for using the double-labelling technique and counts/min were converted to dis/min using appropriate quench correction factors[18]. A Packard Model 3375 liquid scintillation spectrometer was used.

Results. Data shown in Table I indicate that [^{3}H]OH exchanges with brain water to a similar extent in all structures studied except for spinal cord in which much less radioactivity was detected. Substances which enter mainly the extracellular space ([^{3}H] inulin and [^{14}C] sucrose) however, distributed rather evenly in all structures, but there existed a tendency for less radioactivity to be incorporated into hippocampus than in other structures. Carbon atoms of D-glucose were incorporated to a similar extent into all structures studied with the possible exception of spinal cord and those of D-fructose were incorporated to a lesser extent into hippocampus, diencephalon and spinal cord than into other structures (Table II). Incorporation of [^{14}C] of pyruvate did not differ significantly for any brain structure, but the ratio of [^{14}C] of pyruvate to [^{3}H] of inulin in hippocampus (Table III) was markedly higher than that for any other structure. Although the total radioactivity due to [^{14}C] of injected glucose was similar in all brain structures (Table I), determinations of the [^{14}C]/[^{3}H] ratios (i.e., [^{14}C] glucose: [^{3}H]OH) shown in Table III indicated that much less of the total water space was occupied by the [^{14}C] of injected glucose in cerebellum hippocampus and diencephalon than in the other CNS areas studied.

The results presented indicate a number of trends which may prove useful for the further neurochemical analysis of brain structures with respect to behavioral changes brought about by environmental or pharmacological stimuli. Since the exchange of [^{3}H]OH with total brain water was complete within a few min, values in Table I can be taken to represent a comparison of brain structures on the basis of their total water content. For example spinal cord contains less water per g fresh weight than other CNS structures which is known from other studies. Although some loss of water may have occurred during dissection of brain structures this method may prove to be more sensitive than methods based on tissue dry weight for determination of total water content of or changes in water content of brain which may be brought about by pharmacological insult, by dehydration[3] or by other means (e.g. certain nervous and mental diseases).

Although the extracellular space markers (inulin and sucrose) did not equilibrate between brain and blood at 30 min post-injection it seems apparent (Table I) that

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[2] E. A. BERING, J. Neurosurg. *9*, 275 (1952).
[3] F. V. DEFEUDIS, Experientia *27*, 1284 (1971).
[4] J. D. FENSTERMACHER, in Proc. Symp. Blood-Brain Barrier, Wates Fdn. (Ed. R.V. COXON; Truex, Oxford 1970), p. 49.
[5] H. F. BRADFORD, in *Applied Neurochemistry* (Eds. A. N. DAVISON and J. DOBBING; Davis, Philadelphia 1968), p. 222.
[6] J. H. QUASTEL, in *The Structure and Function of Nervous Tissue* (Ed. G. H. BOURNE; Academic Press, New York 1969), vol. 3, p. 61.
[7] F. V. DEFEUDIS, Arch. int. Pharmacodyn. *193*, 322 (1971).
[8] F. V. DEFEUDIS, Life Sci. *10* (Part II), 1187 (1971).
[9] F. V. DEFEUDIS and W. C. BLACK, Expl Neurol. *36*, 41 (1972).
[10] F. V. DEFEUDIS, J. M. R. DELGADO and G. H. ROTH, Nature, Lond. *223*, 74 (1969).
[11] F. V. DEFEUDIS, J. M. R. DELGADO and R. H. ROTH, Brain Res. *18*, 15 (1970).
[12] F. V. DEFEUDIS and R. M. PAOLINO, Experientia *28*, 309 (1972).
[13] M. K. GAITONDE, D. R. DAHL and K. A. C. ELLIOTT, Biochem. J. *94*, 345 (1965).
[14] J. R. LINDSAY and H. S. BACHELARD, Biochem. Pharmac. *15*, 1045 (1966).
[15] W. SACHS, J. appl. Physiol. *10*, 37 (1957).
[16] F. V. DEFEUDIS and K. A. C. ELLIOTT, Can. J. Physiol. Pharmac. *46*, 803 (1968).
[17] F. V. DEFEUDIS, Res. Commun. Chem. Path. Pharmac. *2*, 189 (1971).
[18] F. V. DEFEUDIS, Neuropharmacology *11*, 879 (1972).

Table I. Incorporation of radioactive atoms of water, inulin and sucrose into various structures of mouse brain

Substance injected and dose	Radioactivity in homogenate; dis/min/g fresh weight ($\times 10^{-3}$)						
	Cerebral cortex	Cerebellum	Olfactory bulbs	Hippocampus	Diencephalon	Spinal cord	Colliculi
^{3}HOH (0.25 μCi/g)	389.6 ± 17.09 (18)	440.8 ± 11.04 (18) [a]	388.8 ± 19.51 (18)	400.2 ± 13.16 (18)	413.6 ± 8.83 (18)	323.9 ± 16.32 (17) [b]	441.5 ± 11.07 (6)
^{3}H-inulin (0.25 μCi/g)	31.9 ± 0.86 (5)	33.0 ± 1.40 (5)	33.7 ± 4.30 (5)	29.7 ± 0.95 (5)	30.2 ± 1.36 (5)	31.5 ± 2.80 (5)	—
U-^{14}C-D-sucrose (0.1 μCi/g)	12.9 ± 3.37 (6)	13.1 ± 3.29 (6)	16.7 ± 3.24 (6)	10.8 ± 3.56 (6)	11.1 ± 2.68 (6)	17.8 ± 3.92 (6)	—

All animals were sacrificed 30 min after s.c. injection of radioactive substances. Means ± S.E.M.; numbers of animals in parentheses. [a] and [b] indicate $p < 0.02$ and $p < 0.001$, respectively when these values are compared with that for cerebral cortex (Student's t-test; 2-tailed).

Table II. Incorporation of radioactive carbon atoms of glucose, fructose and pyruvate into various structures of mouse brain

Substance injected and dose	Radioactivity in homogenate; dis/min/g fresh weight ($\times 10^{-3}$)						
	Cerebral cortex	Cerebellum	Olfactory bulbs	Hippocampus	Diencephalon	Spinal cord	Colliculi
U-^{14}C-D-glucose (0.1 μCi/g)	451.5 ± 54.91 (6)	438.9 ± 37.90 (6)	427.2 ± 38.44 (6)	404.3 ± 38.22 (6)	394.5 ± 32.37 (6)	316.8 ± 57.03 (5)	—
U-^{14}C-D-fructose (0.05 μCi/g)	158.5 ± 11.65 (6)	155.8 ± 6.01 (6)	155.7 ± 7.22 (6)	135.7 ± 3.85 (6) [a]	138.3 ± 3.00 (6) [a]	111.0 ± 5.15 (6) [b]	171.1 ± 4.10 (6)
1-^{14}C-pyruvate (0.05 μCi/g)	14.2 ± 0.58 (5)	15.2 ± 0.73 (5)	14.7 ± 1.02 (5)	16.1 ± 0.91 (5)	14.2 ± 0.33 (5)	14.6 ± 0.35 (5)	—

All animals were sacrificed 30 min after s.c. injection of radioactive substances. Means ± S.E.M.; numbers of animals in parentheses; [a] and [b] indicate $p < 0.05$ and $p < 0.001$, respectively, when these values are compared with that for cerebral cortex (Student's t-test; 2-tailed).

Table III. Ratios of ^{14}C/^{3}H of radioactive substances incorporated into various structures of mouse brain using the double-labelling technique

Substances injected and doses	^{14}C/^{3}H ratio, based on dis/min/g fresh weight						
	Cerebral cortex	Cerebellum	Olfactory bulbs	Hippocampus	Diencephalon	Spinal cord	Colliculi
U-^{14}C-D-glucose, 0.1 μCi/g + ^{3}HOH, 0.25 μCi/g	1.42 ± 0.096 (6)	1.03 ± 0.067 (6) [b]	1.30 ± 0.066 (6)	1.09 ± 0.076 (6) [a]	1.00 ± 0.070 (6) [b]	1.31 ± 0.155 (5)	—
U-^{14}C-D-fructose, 0.05 μCi/g + ^{3}HOH, 0.25 μCi/g	0.35 ± 0.019 (6)	0.32 ± 0.016 (6)	0.32 ± 0.014 (6)	0.30 ± 0.009 (6)	0.31 ± 0.015 (6)	0.30 ± 0.011 (6)	0.39 ± 0.010 (6)
U-^{14}C-D-sucrose, 0.1 μCi/g + ^{3}HOH, 0.25 μCi/g	0.03 ± 0.008 (6)	0.03 ± 0.008 (6)	0.05 ± 0.007 (6)	0.03 ± 0.010 (6)	0.03 ± 0.007 (6)	0.05 ± 0.011 (6)	—
1-^{14}C-pyruvate, 0.05 μC-/g + ^{3}H-inulin, 0.25 μCi/g	0.45 ± 0.011 (5)	0.46 ± 0.01 (5)	0.45 ± 0.040 (5)	0.54 ± 0.013 (5) [c]	0.47 ± 0.014 (5)	0.48 ± 0.037 (5)	—

All animals were sacrificed 30 min after s.c. injections of doubly-labelled solutions. Means ± S.E.M.; numbers of animals in parentheses; [a], [b] and [c] indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, when these values are compared with those for cerebral cortex (Student's t-test; 2-tailed).

no major difference in their distribution exists among the brain parts studied with the possible exception of the hippocampus which may have a smaller 'effective' extracellular space. Hence these substances can serve as reference or 'control' substances. By comparing values for [^{14}C] glucose (Table II) with those for [^{3}H]OH (Table I) it becomes apparent that in all structures studied incorporation of [^{14}C] of glucose is a function of the total water content, but comparisons between [^{14}C] fructose and [^{3}H]OH reveal that diencephalon and hippocampus contain less [^{14}C] than would be expected from analysis of the [^{3}H]OH data. This may be related to different rates of fructose metabolism in different cerebral structures.

Ratios of [^{14}C]/[^{3}H] of simultaneously-injected radioactive glucose and water (Table III) indicate that certain brain structures (i.e., cerebellum, hippocampus and diencephalon) can be shown to differ from the others by this method. Less [^{14}C] of glucose entered these structures than was expected on the basis of [^{3}H]OH exchange which may indicate that energy metabolism occurs to a lesser extent (or more slowly) in these regions. The finding that [^{14}C] atoms of fructose sucrose or pyruvate with the exception of hippocampus were incorporated to similar extents into all structures with respect to [^{3}H] (of [^{3}H]OH or [^{3}H] inulin) indicates that incorporation of these substances is related directly to the 'spaces' of brain or to its total water content. This method might be useful for studying changes in the metabolism of energy-yielding substrates which may be produced in awake behaving animals by pharmacological insult or environmental stimuli. However, it must be realized that the incorporation into brain of substances which can be metabolized is subject to diurnal variation[19,20] and therefore that time of day is a critical factor in this type of study.

Résumé. Une méthode nouvelle est présentée pour étudier des corrélations qui existent entre le cerveau et le comportement des animaux.

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[19] A. SOLLBERGER. Suppl. Rep. 5th Int. Conf. Biol. Rhythm, Stockholm, (1960), p. 111.
[20] A. SOLLBERGER, *Biological Rhythm Research* (Elsevier, Amsterdam 1965), p. 461.

Untersuchungen über Ultrastruktur und Aminosäurenzusammensetzung der Eischalen von *Natrix natrix*

Die Eischalen von vielen Reptilien – insbesondere der Schlangen – sind nicht eindeutig verkalkt, auch wenn bei *Natrix natrix natrix* gelegentlich aufgelagerte Kalkkristalle beobachtet werden[1,2]. Im Gegensatz zu Vogeleischalen finden sich in nicht kalkigen Eischalen von Reptilien völlig anders aufgebaute Proteine[3]. Innerhalb der Reptilien zeigt sich aber auch bei nicht verkalkten Eischalen eine deutliche Gruppierung auf Grund einer unterschiedlichen Aminosäurenzusammensetzung. Bisher konnten mindestens 4 typische Aminosäuren-Profile festgestellt werden: Dermochelys-Typ, Tarentola-Typ, Phelsuma-Typ und Eublepharis-Typ[2].

Die in Eischalen von Lederschildkröten (*Dermochelys coriacea*) gefundenen Aminosäuren-Relationen unterscheiden sich sehr deutlich von den 3 übrigen bei Gekkos auffallenden Aminosäuren-Verhältnissen. Das erstmalig bei *Eublepharis* festgestellte Aminosäuren-Profil ist charakteristisch für praktisch alle Squamaten[2]. Auch lassen sich bei kalkigen Schalen von Reptilien unterschiedliche kristalline Bausteine ermitteln[4].

Ziel dieser Arbeit war es, Eischalen von Schlangen (*Natrix natrix natrix*) in ihrer Struktur und ihrer Aminosäuren-Zusammensetzung zu untersuchen und mit bereits bekanntem Material zu vergleichen.

Versuchsmethodik. Frische Schalen von Eiern der Ringelnatter und der Membrana testacea des Haushuhns (HNL-Hybridhuhn) wurden in eine 1%ige Osmiumsäurelösung (0,1 *M* Cacodylat-Puffer mit 7,5% Sucrose, pH 7,2, Osmolarität 450) 2 h fixiert und nach Dehydrierung mit Alkohol in Epon eingebettet. Bleinitrat diente zur Nachkontrastierung der Dünnschnitte. Die Färbung mit Rutheniumrot (modifiziert nach LUFT[5]) erfolgte bei der Vorfixierung in 3% Glutaraldehyd (0,1 *M* Cacodylat-Puffer mit 0,5% Sucrose, pH 7, 2, 2000 ppm Rutheniumrot). Die weiteren Schritte wurden entsprechend der oben beschriebenen Methode (aber ohne Nachkontrastrierung) durchgeführt.

Die Aminosäuren-Analyse erfolgte an einem entsprechenden Analysator nach Hydrolyse in 6 *N* HCl (24 h) bei 110°C[6]. Zur Cystin-Bestimmung haben wir die Proben nach HIRS[7] mit Perameisensäure vorbehandelt.

Ergebnisse und Diskussion. Die Eischalen von Ringelnattern sind aus Fasern aufgebaut, die untereinander in Verbindung stehen (Figur 1). Jede Faser besteht aus einer weniger elektronendichten Hülle (mit feiner, granulärer Textur) und der homogenen Core-Substanz (Medulla-Substanz). Das Core ist von zahlreichen Hohlräumen – besonders bei den Fasern im äusseren Bereich der Schale – durchsetzt. In der Feinstruktur ähneln diese Fasern denen der Membrana testacea vom Haushuhn (Figur 2)[8], jedoch ist die Hüllsubstanz bei der Ringelnatter (0,15 µm) schwächer ausgebildet als beim Haushuhn (0,3 µm). Die Faserhüllen lassen sich – besonders in der äusseren Zone – bei der Ringelnatter und beim Huhn mit Rutheniumrot tingieren (Figuren 1a und 2a).

Die Core-Substanz ist dagegen nicht färbbar. Die Ruthenium-Reaktion in der Faserhülle der Ringelnatter

[1] H. K. ERBEN, persönliche Mitteilung.
[2] G. KRAMPITZ, W. BOEHME, K. KRIESTEN und H. HARDEBECK, Z. vergl. Zool. Evolutionsforsch., im Druck.
[3] G. KRAMPITZ, K. KRIESTEN und R. FAUST, Biomineralisation, im Druck.
[4] G. KRAMPITZ, H. K. ERBEN und K. KRIESTEN, Biomineralisation *4*, 88 (1972).
[5] J. K. LUFT, Anat. Rec. *171*, 347 (1971).
[6] G. KRAMPITZ und J. WIENEKE, Z. Tierphysiol. Tierernähr. Futtermittelk. *18*, 147 (1963).
[7] C. H. W. HIRS, Meth. Enzym. *11*, 197 (1967).
[8] C. M. SIMONS, und G. WIERTZ, Z. Zellforsch. *59*, 555 (1968).