INCORPORATION OF ^{18}O INTO HOMOVANILLIC ACID OF RAT BRAIN DURING EXPOSURE TO OXYGEN 18 CONTAINING ATMOSPHERES 1)

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SUMMARY

Rats were exposed to air containing $^{18}O_2$ at atmospheric pressure. In vivo incorporation of ^{18}O in brain homovanillic acid (HVA) was determined by gas chromatography-mass spectrometry. One ^{18}O atom was incorporated into each molecule of HVA indicating that tyrosine is the predominant precursor of brain dopamine and that the oxygen in the 3-position is of atmospheric origin. Intraperitoneal administration of ^{18}O-enriched water did not alter the ^{18}O content of brain HVA. Mass fragmentography (2) was used to measure the increase in ^{18}O and the decrease in ^{16}O in HVA from rat brain over several hours of exposure to an ^{18}O enriched atmosphere. These experiments demonstrate the possibility to pulse label brain dopamine and its metabolites by in vivo inhalation of stable oxygen isotopes. The procedure should be useful for quantitative determinations of the turnover of brain dopamine in animals and man.

Homovanillic acid (HVA) is the main metabolite of dopamine which is a putative transmitter substance in the central nervous system (1). Dopamine is formed from tyrosine and possible from phenylalanine in specific brain neurons in reactions requiring tyrosine 3-hydroxylase (EC 1.10.3.1), molecular oxygen and a reduced pteridine cofactor (2, 3). The product, dihydroxyphenylalanine (DOPA), is then rapidly decarboxylated to dopamine. A number of techniques have been developed for the quantitative determination of dopamine turnover in animal brain (4). The methods to date involve pharmacological agents or labelling of brain dopamine by the administration of radioactive precursors. The fact that the enzymes in catecholamine biosynthesis utilize molecular oxygen

1) A preliminary report of the present work was presented at the "Animal Pharm" meeting, Uppsala, Sweden, June 1972.

(8) opens the possibility of labelling catecholamine in vivo by a single exposure of the organism to an atmosphere enriched in a stable isotope of oxygen and of detecting such labelled compounds by the recently developed gas chromatographic-mass spectrometric systems (5,6,7). It should, in principle, be possible to label several compounds of biological interest in this way. The present study is the first to examine this approach and demonstrate the in vivo incorporation of ^{18}O into the HVA of rat brain. The procedure has potentially broad application. Further, since neither potent pharmacological agents nor radioactive materials are employed, the procedure may be particularly fruitful for studies in man.

MATERIALS AND METHODS

A. Exposure of rats to $^{18}O_2$.

Oxygen gas, highly enriched with $^{18}O_2$, was prepared by electrolysis of ^{18}O-enriched water as described by Samuel (9). ^{18}O-enriched water containing 90-96 atom per cent was obtained from the Isotope Separation Plant of the Weizmann Institute of Science, Rehovot, Israel.

Sprague-Dawley rats (CR strain) weighing 40-60 grams were maintained in a closed system at atmospheric pressure in which atmospheric oxygen was slowly enriched with $^{18}O_2$. This was accomplished by use of an electronic valve operated by a mercury manometer which introduced $^{18}O_2$ into the system as oxygen was consumed and the resultant CO_2 was removed by absorption onto KOH and silica gel. The air in the system was kept in constant circulation by means of a peristaltic pump. Gas samples were removed at regular intervals for determination of the ^{18}O content by mass spectrometry. After periods of up to 3 hours animals were killed

by decapitation; the brains were removed and homogenized in 40% ethanol for determinations of the isotopic content of HVA. A sample of blood was also taken from each animal for isotopic analysis. A schematic representation of the apparatus is shown in Fig. 1.

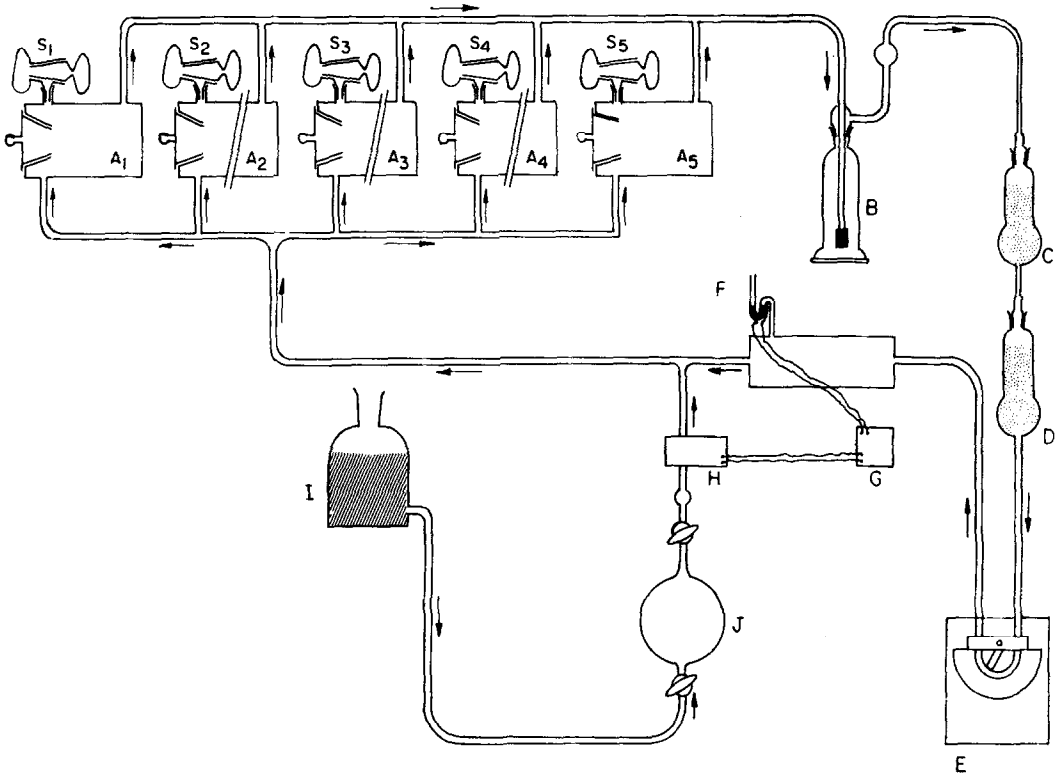

Fig. 1 Apparatus for maintaining rats in a controlled atmosphere at atmospheric pressure. A_1-A_5 - rat chamber, B - Washbottle containing conc. KOH for removed CO_2, C, D - silical gel, drying tubes, E - peristaltic pump, F - mercury manometer, G - relay, H - electric valve, J - 5 litre flask containing highly enriched $^{18}O_2$, I - water pressure head, S_1-S_5 - evacuated bulbs for sampling circulating atmosphere.

B. Injection of rats with $H_2{}^{18}O$.

Rats were injected i.p. with 0.25 ml water, containing either

99.8% ^{16}O or 98% ^{18}O. After three hours the animals were killed, their brains removed and homogenized in 40% ethanol for the determination of the isotopic content of HVA. A sample of blood was also taken from each animal for isotopic oxygen analysis.

C. Mass spectrometric determinations.

The analysis of $^{18}O_2$ in the atmosphere was performed by direct mass spectrometry of samples taken at given times using a MAT Varian CH-7 mass spectrometer. The analysis of the ^{18}O in blood was performed by distilling the water from whole blood samples and equilibration with CO_2 and subsequent mass spectrometry as described by Samuel (9). HVA was extracted from the brain homogenate as described by Sjöquist et al. (7). HVA was then converted to the methyl ester, deuterated methyl ester of HVA was added as both carrier and internal standard and the heptafluorobutyrate derivatives synthesized. Samples of the HVA derivatives were injected into an LKB 9000 gas chromatograph-mass spectrometer, equipped with a 1% SE-30 or a 1% OV-17 column. The instrumental conditions were the same as previously described (7). Mass spectra of HVA derivatives obtained from the brain of rats exposed to $^{16}O_2$ and $^{18}O_2$ atmospheres were compared to those of authentic samples of HVA derivatives. The spectra were obtained by injecting approximately 80 ng of the compound. The HVA derivatives had a retention time of 4.20 min on the OV-17 column. For routine analysis brain extracts were divided into two parts which were processed with or without the deuterated internal standard. The mass spectrometer was equipped with an accelerating voltage alternator (AVA) which allows the simultaneous recording of two mass numbers. Since the molecular ion peak of HVA methyl ester heptafluorobutyrate, m/e 392, is also the base peak concentra-

tions were obtained by comparing intensities of the molecular ions of the natural (^{16}O), heavy oxygen (^{18}O) and deuterium (D_3) forms of the HVA derivative at m/e values 392, 394 and 395 respectively. By relating these peak ratios to a standard curve obtained from mixtures of the deuterium standard with increasing amounts of ^{16}O HVA, the absolute amounts of ^{16}O- and ^{18}O-HVA in extracts can be calculated and expressed in picomoles per brain (7). The brain weight of the rats used was about 1.2 g.

RESULTS

A. Mass spectrometric identification of ^{18}O labelled HVA formed in rat brain following in vivo exposure to $^{18}O_2$.

Rats were placed in the exposure chamber and were maintained in an atmosphere containing either $^{16}O_2$ or $^{18}O_2$ for 3 hours. Fig. 2 demonstrates the high mass range in the mass spectra of the methyl ester heptafluorobutyryl derivative of authentic HVA and apparent HVA from brain extracts. HVA has a base peak at m/e 392 which is also the molecular ion. An abundant fragment is also present at m/e 333. An almost identical spectrum was obtained from brain extracts or animals exposed to an $^{16}O_2$ containing atmosphere. On the other hand, in animals exposed to $^{18}O_2$ gas, abundant fragments at m/e 394 and 395 were also present, demonstrating that the HVA contained one ^{18}O atom per molecule. A small fragment was obtained at m/e 396 but not at 337 which indicates that HVA with two $^{18}O_2$ atoms is not formed to any appreciable extent. The absolute amounts of ^{16}O and ^{18}O containing HVA were calculated from peak height ratios of extracts with and without the deuterated methyl ester of HVA. The data in Table 1 demonstrate the marked increase in ^{18}O-labelled HVA and an approximately corresponding decrease in the amount of ^{16}O-labelled HVA. As demonstrated previously (10) in vivo exposure to $^{18}O_2$ containing

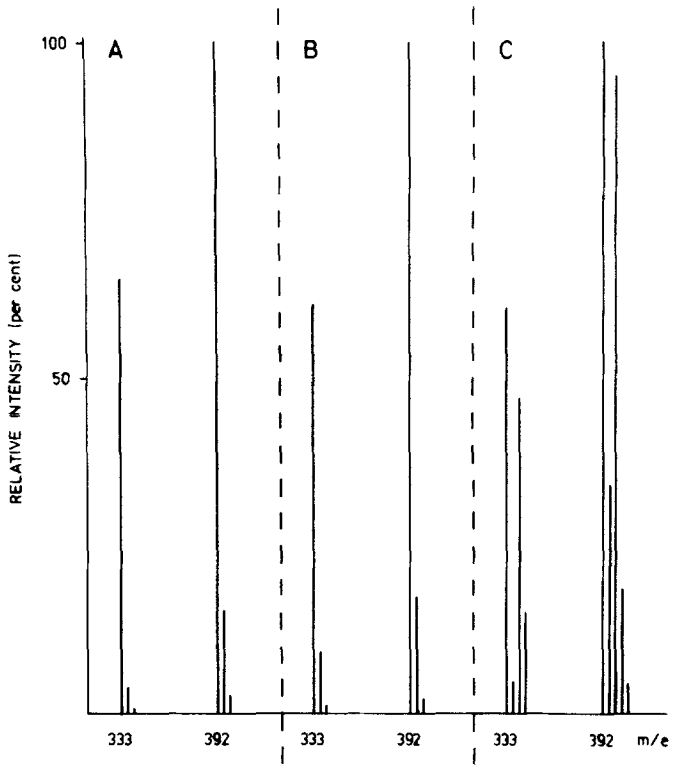

Fig. 2 High mass range of HVA in the form of Me-HFB derivatives. A represents authentic HVA, B and C are obtained from brain of rats exposed to $^{16}O_2$ and $^{18}O_2$ respectively. Spectra were obtained by subtracting background intensities at retention time 4.15 min from values recorded at 4.20 min - the retention time of the HVA derivatives on the 1% OV-17 column used.

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atmospheres results in an increase in the amount of ^{18}O in body water.

B. Evidence for atmospheric origin of ^{18}O in HVA.

Rats were injected i.p. with $H_2{}^{16}O$ or $H_2{}^{18}O$. Three hours later they were killed and the levels of ^{16}O and ^{18}O HVA and the atom per cent ^{18}O in serum water was determined. The amount of ^{18}O in serum was significantly higher in the animals injected with ^{18}O-labelled water. There was no significant difference between the two groups in the content of ^{16}O labelled HVA in the brain (Table 1). Moreover, significant amounts of ^{18}O-labelled HVA

Table 1

OXYGEN ISOTOPIC LEVELS OF HOMOVANILLIC ACID IN RAT BRAIN AFTER EXPOSURE TO $^{16}O_2$ OR $^{18}O_2$ LABELLED AIR OR i.p. INJECTIONS OF ^{16}O OR ^{18}O-LABELLED WATER

Treatment	^{16}O-HVA pmole/brain	^{18}O-HVA pmole/brain	Atom percent ^{18}O
$^{16}O_2$ exposure	1215 ± 224	not dectected	0.204
$^{18}O_2$ exposure	524 ± 61[2)]	649 ± 90	1.356[1)] ± 0.045
$H_2{}^{16}O$ injection	1182 ± 61	not detected	0.204
$H_2{}^{18}O$ injection	1163 ± 41	not detected	0.938[1)] ± 0.019

Data represent mean ± s.e. from 3-4 rats. 1) $p < 0.001$ 2) $p < 0.05$

were not formed in brain following neither treatment.

C. Time course for accumulations of ^{16}O and ^{18}O HVA in rat brain following exposure to $^{18}O_2$ or $^{16}O_2$.

In Fig. 3 changes in the ^{18}O and ^{16}O content of HVA following exposure for various lengths of time to $^{18}O_2$ and $^{16}O_2$ containing air is presented. Following exposure to $^{18}O_2$, the level of $^{16}O_2$ HVA rapidly declined and was replaced by approximately the same amount of ^{18}O HVA. After interruption of exposure the reversed changes took place. No significant amounts of ^{18}O HVA were found when animals were exposed to an ^{16}O-containing atmosphere for 3 hours.

DISCUSSION

Previous studies on the enzymatic hydroxylation of tyrosine have demonstrated the atmospheric origin of the oxygen in the 3-position of the product DOPA (2,8). The mass spectra presented in

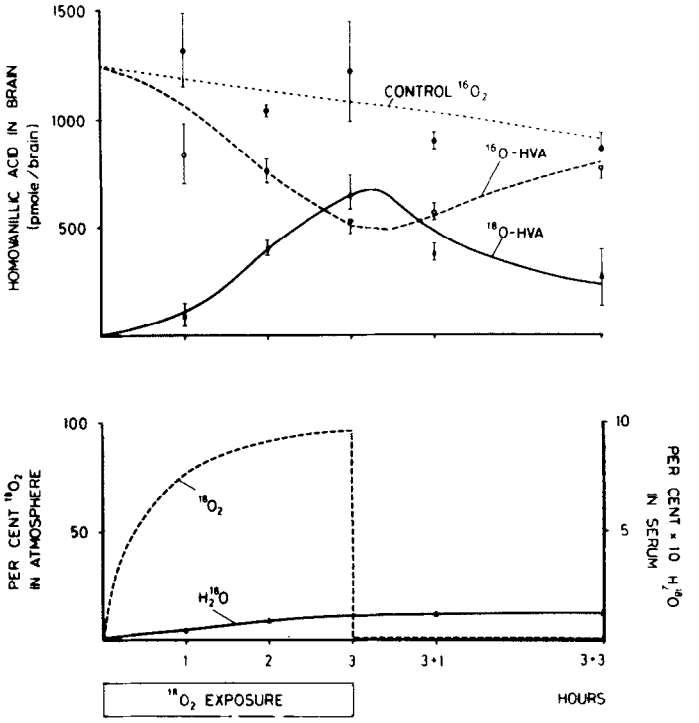

Fig. 3 Time course for changes in levels of ^{16}O-HVA, ^{18}O-HVA, $H_2{}^{18}O$ and $^{18}O_2$ during exposure of rats to an $^{18}O_2$ containing atmosphere.

Fig. 2 is the first in vivo confirmation of this, and shows that HVA from brains of $^{18}O_2$-exposed animals contains one ^{18}O atom per molecule. As found previously (10) the rate of formation of ^{18}O-labelled water in the blood during exposure to highly enriched $^{18}O_2$ was relatively low and did not exceed 1.5 atom per cent ^{18}O in any of the experiments. The i.p. injection of ^{18}O-labelled water resulted in about the same percentage ^{18}O in body water, but caused no significant ^{18}O-labelling of HVA in the brain. Moreover, ther was no relation in the kinetic experiment between the rate of accumulation of ^{18}O HVA and $H_2{}^{18}O$ (Fig. 3) which also demonstrates that the incorporated ^{18}O atom derives solely from molecular oxygen. No evidence for the presence of

TABLE 1

External $[Ac^-]$ (mM)	Internal Free Mn^{++} (nMoles/mg)	Internal $[Mn(H_2O)_6^{++}]$ (mM)
0	2.89	8.28
2.5	4.47	12.2
10	4.85	12.7
30	9.62	20.7
100	18.35	58.7

Table 1. Before addition of EDTA each sample (2ml total volume) contained 11.7 mg mitochondrial protein, 10mM Tris-Cl (pH 7.4), 10mM sodium succinate, .1mM ATP, .1% BSA, and 2 μMoles $MnCl_2$ and varying amounts of sucrose and sodium acetate. After allowing 15 minutes for uptake 2.5μMoles EDTA was added and the EPR spectrum taken. The external osmolarity was ≃ .28M except for the 100mM $[Ac^-]$ sample where it was .43M. Protein was determined by the Biuret Method.

lower. Combined with the results in Table 1, it appears that the mitochondria can maintain a concentration gradient of at least 500:1. A comment on activity coefficients is appropriate here. Because of the high concentration of phospholipids, small anions, and proteins, some of which bind Mn^{++}, the average activity coefficient for all the Mn^{++} transported is expected to be low. The narrow line-width (30-35 Oe) of S indicates that this spectral component arises from a population of ions, which during the characteristic spin state lifetime ($T_1 \simeq T_2$), are not immobilized or in strong association with negative groups. Each ion has a full complement of six waters of hydration in its first coordination shell and has no negative groups disturbing its second coordination shell except for a possible rapid exchange of singly charged anions (9, 10). The water molecules outside the first hydration sphere must be highly mobile (10, 11). The average activity coefficient for this fraction of Mn^{++} should be much higher (of order unity). Moreover, weakly bound fractions of Mn^{++} may exist that contribute to the activity but not to S. It is therefore conceivable that S could even under-

instance, that chlorpromazine treatment markedly accelerates the *in vivo* accumulation of ^{18}O HVA - labelled in brain (14). The possibility of labelling brain HVA by the present method should be of considerable value in quantitative analysis of rates of brain catecholamine synthesis in both animals and man. A distinct advantage of this method is that by inhalation the molecular oxygen can freely enter the brain via the blood stream and is not hampered by the restrictions imposed by the blood-brain barrier. Since a number of organic compounds of biological interest are formed by hydroxylases using molecular oxygen, the present technique should also be useful for the study of other metabolic pathways *in vivo*.

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