Cerebral Metabolism of L-[2-^{18}F]Fluorotyrosine, a New PET Tracer of Protein Synthesis

Heinz H. Coenen, Peer Kling, and Gerhard Stöcklin

Institute of Chemistry 1 (Nuclear Chemistry), Nuclear Research Centre Jülich GmbH, Jülich, FRG

L-[2-^{18}F]fluorotyrosine (2-^{18}FTyr) was evaluated as a tracer of cerebral protein synthesis for positron emission tomography (PET). Its metabolism in murine cerebrum was studied. The uptake in brain reaches a value of ~2% of the injected dose per gram tissue after 60 min. The incorporation of the tracer into tissue proteins was proven by discontinuous SDS gel electrophoresis. The protein bound fraction of tissue activity increased to 84% and 89% after 60 and 120 min p.i., respectively. High performance liquid chromatography analysis showed a concomitant decrease of free 2-^{18}FTyr in tissue with time. The sum of free 2-^{18}FTyr, tRNA- and protein-bound 2-^{18}FTyr in cerebral tissue gave an almost quantitative activity balance of 96 ± 4% at all times examined. A significant formation of fluorodopa or fluorodopamine must therefore be excluded. This shows that L-[2-^{18}F]fluorotyrosine is a promising tracer for quantitation of protein synthesis rates with PET based on a three-compartment model.

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In the physiology of the body, the turnover of amino acids and proteins represents a major biochemical process. The quantitative assessment of cerebral protein synthesis in vivo under normal and pathologic conditions is of special interest (*1*). Positron emission tomography (PET) principally allows quantitation of metabolic rates when suitable positron emitter labeled tracers are available. Attempts have been undertaken to develop strategies and to establish appropriate tracer kinetic models for quantitation of protein synthesis (*2, 3*). Almost exclusively, carbon-11 (^{11}C) ($T_{½}$ = 20 min) labeled natural amino acids have been used for this purpose. Especially L-[1-^{11}C]leucine, L-[^{11}C-methyl]methionine, and more recently L-[1-^{11}C]tyrosine were applied to study primarily tumor metabolism (*3–6*).

One disadvantage of using ^{11}C-labeled amino acids is that the half-life of ^{11}C is short in comparison to the relatively slow protein synthesis rates (PSR) in primate brain that requires ≥60 min (*7*). Furthermore, natural amino acids enter metabolic pathways besides protein synthesis, requiring the inclusion of four or even more compartments into the kinetic model. In the case of leucine (*3*), the rapid loss of [^{11}C]CO_2 from the C_1-position has to be considered. However, it was recently claimed that this process is negligible for L-[1-^{11}C]-tyrosine, making this tracer an exception (*8*). Labeling of amino acids in positions other than the carboxylic group, as recently done for [3-^{11}C]-phenylalanine (*9*), will also yield labeled metabolites after decarboxylation. In the case of L-methionine, the methyl group is a source of methylation in the biochemistry of cells. Significant levels of labeled metabolites were already found in blood plasma 15 min after i.v. administration of L-[^{11}C-methyl]methionine (*10,11*). At early times, uptake kinetics primarily reflect the transport of amino acids into the tissue (*12*) and hence the determination of PSR is rather uncertain. Consequently, L-[^{11}C]methionine was recently used for the assessment of amino acid transport measurements in the human brain (*13*).

We have studied analogous amino acids, especially radiohalogenated aromatic ones, as potential tracers of protein synthesis (*14*). These are chemically stable compounds with a relatively small molecular change when compared to the original molecules. Substrates labeled with the PET nuclide fluorine-18 (^{18}F) are particularly likely candidates because of the convenient half-life ($T_{½}$ = 110 min) and the small sterical changes. In fact, fluoroaromatic amino acids have been a focus of interest in physiology for many years (*15*). For para-fluorophenylalanine, an effective incorporation into proteins was found (*16*) and its potential usefulness for PET was recently demonstrated (*17*). However, this compound, which has also recently been prepared by a multistep

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For reprints contact: H. H. Coenen, PhD, Institut für Chemie 1 (Nuklearchemie), Kernforschungsanlage Jülich GmbH, D-5170 Jülich.

radiosynthesis (*18*), exhibits a rather slow protein incorporation (*14*) which is also dependent on the concentration of L-phenylalanine (*16*).

No general prediction can be made on the physiologic acceptance of amino acid analogs. Very different pharmacologic effects and toxicities were already observed for positional isomers of fluoroaromatic amino acids (*15*). In contrast to natural amino acids, the detailed physiology of most analogs is unknown and the biochemical behavior must be determined in each case. We have recently demonstrated in preliminary screening experiments that besides p-fluorophenylalanine only L-[2-^{18}F]-fluorotyrosine (2-^{18}FTyr) is incorporated into cerebral proteins of mice (*14*). Although there are biochemical reports on the isomers of fluorophenylalanine and 3-fluorotyrosine (*15*), nothing so far is known about the biochemistry of 2-fluorotyrosine. An indication for its acceptance in protein synthesis in cerebrum was found in a previous study on ortho-[^{18}F] fluorophenylalanine after the administration of which 2-fluorotyrosine was found in the aminoacylated tRNA pool (*17*). A detailed study of the cerebral biochemistry of 2-^{18}FTyr in mice with special emphasis on protein incorporation is reported here. Discontinuous SDS gel electrophoresis was applied to prove its acceptance by the aa-tRNA synthetase and general incorporation into proteins in vivo. Other possible metabolic transformations are also investigated.

MATERIALS AND METHODS

[^{18}F]F_2 was produced via the ^{20}Ne(d,α)^{18}F reaction on a neon/0.1% F_2 gas mixture and used directly in its elemental form or after conversion to [^{18}F]acetylhypofluorite for electrophilic radiofluorination of O-acetyltyrosine in CF_3CO_2H as solvent at 0°C (cf. Scheme 1). The desired product L-[2-^{18}F] fluorotyrosine was isolated from the isomeric mixture obtained after base catalyzed hydrolysis of the acetyl moiety by means of reverse phase HPLC. Nuclide production, precursor synthesis from tyrosine, radiofluorination and chromatographic methods including preparation of the cold standard compound have been described elsewhere in detail (*19*). In contrast to a fluorination in liquid HF as solvent with only 3% radiochemical yield (*20*), a 17% yield was obtained in CF_3CO_2H with a specific activity of 10 to 20 GBq/mmol. A still higher regioselective yield can be expected from fluorodemetallation reactions presently examined. L-Tyrosine, other reagents and solvents were used as obtained commercially from Merck, Darmstadt FRG. A study on acute toxicity of D,L-2-fluorotyrosine with doses up to 30 mg/kg body weight showed no behavioral or histologic effects in mice (carried out by Grünenthal GmbH, Aachen, FRG).

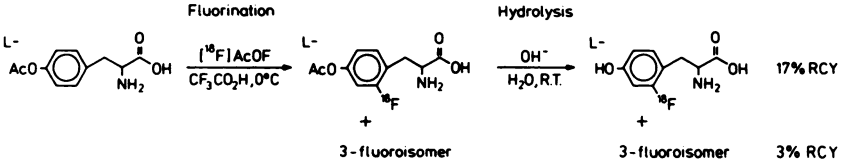
For measurements of cerebral uptake and protein incorporation in vivo, L-[2-^{18}F]fluorotyrosine was administered to NMRI mice (~30 g) via tail vein injection. About 1 MBq of the tracer (~5 μg) was administered in 0.1 ml of physiologic saline solution. Groups of five to eight animals were killed after 5, 10, 20, 40, 60, or 120 min postinjection. Brains were quickly removed within 20 sec and frozen in liquid nitrogen. Blood samples were also taken at these times. Weighed blood and brain tissue samples were measured for total radioactivity in a well-type scintillation counter.

Frontal parts of cortex tissue (~50 to 100 mg wet weight) were homogenized by addition of 1 ml distilled water and by ultrasonication at 0°C. Proteins were precipitated and isolated by addition of trichloroacetic acid (TCA) up to a final concentration of ≥10%, centrifugation, removal of the supernatant and twofold washing of the precipitate with 10% TCA solution. Radioactivities contained in the acid solution and protein pellet were determined. The supernatant was further analyzed for acid soluble organic fluorocompounds, especially unchanged 2-^{18}FTyr, by means of isochratic high performance liquid chromatography (HPLC) methods as used for the radiosynthesis of the compound (*19*). An aminex anion exchange column was used with 1 N NaOH as eluant to test for free [^{18}F]fluoride. Similarly blood samples were analyzed for free fluoride, protein bound and free L-2-[^{18}F]fluorotyrosine. For determination of the aminoacylated tRNA (aa-tRNA) fraction of 2-^{18}FTyr the brain tissue homogenate was extracted with water-saturated phenol and the aa-tRNA precipitated with 80% ethanol in the cold (*21*). The radioactivity was determined after centrifugation and separation of the precipitate.

To characterize the general incorporation of 2-^{18}FTyr into cerebral proteins ~20 MBq were administered with an incubation time of 40 min. In this case the protein precipitates containing the ^{18}F radioactivity were further separated for their molecular weight by a discontinuous gel electrophoresis method (*22*). Therefore, 0.5 to 0.7 mg of the TCA precipitated proteins were redissolved in 10% sodium dodecylsulfate (SDS) buffer containing bromophenol blue to mark the progress of electrophoresis. This was loaded on polyacrylamide gel columns of 0.5 cm diameter and ~11 cm length in glass tubes. Control gels were loaded with protein standards of known molecular mass. Electrophoresis was performed parallel on 20 SDS gels within 3 hr with a maximum voltage of 140 V and current of 3 mAmp per gel. After development the gels were stained with Coomassie Blue R-250 by standard procedures (*22*), monitored for density by uv absorption and cut in 0.4-cm segments which were measured for radioactivity in a well counter.

RESULTS

The pharmacokinetics of 2-^{18}FTyr in the brain of mice showed an uptake of 1.3 ± 0.05% of injected dose



SCHEME 1
Radiosynthesis of L-[2-^{18}F]fluorotyrosine

per gram tissue at 40 min that increases to 2.1 ± 0.4 and 1.8 ± 0.4 at 60 and 120 min, respectively. Trichloroacetic acid precipitation of homogenized cortex samples showed a steadily increasing fraction of protein bound L-[2-^{18}F]fluorotyrosine reaching 84 ± 1.2% and 88.7 ± 1.5% of total tissue activity after 60 and 120 min, respectively. These data are graphically shown in Figure 1 together with the time course of the free tracer concentration in the tissue as determined by HPLC. No free [^{18}F]fluoride was found in brain tissue.

At 20 and 40 min, times of rapidly increasing protein incorporation (cf. Fig. 1), the tRNA bound fraction of 2-^{18}FTyr was also determined. Phenol extraction and subsequent ethanol precipitation of cortex tissue homogenates revealed only small amounts of 2-^{18}FTyr aminoacylated with tRNA (2-^{18}FTyr-tRNA) with 1.8 ± 0.5% and 1.6 ± 0.7% of tissue activity, respectively. Thus, the percentage of identified tissue activity was almost quantitative (97 ± 5.0%).

The TCA precipitate was further analyzed by means of SDS gel electrophoresis. Cortical proteins of five different animals obtained 40 min p.i. were separated by their electrophoretic mobility as shown for one sample in Figure 2. Comparison of uv density, radioactivity, and mobility of standard proteins with known molecular mass indicated distribution of the radiotracer over all protein sizes with maxima at ~20,000, 50,000, and 100,000 D.

The HPLC analysis of the supernatant of the TCA precipitation of cortex tissue showed one small unknown peak of 0.3 to 1.5% of total tissue activity. This was eluted before 2-fluorotyrosine; i.e., was more polar. However, because of the superposition of much uv-absorbing material, the identity of this labeled product could not be unambiguously clarified. Analogous analysis of striatum tissue, done after 50 min of incubation time, revealed with 23.5% a slightly increased fraction of free 2-^{18}FTyr by ~3% while protein binding with 69.3% was ~5% lower than that of cortex tissue. The radioactivity of the unknown peak with the same retention as from cortex tissue was increased to 4.2%. But

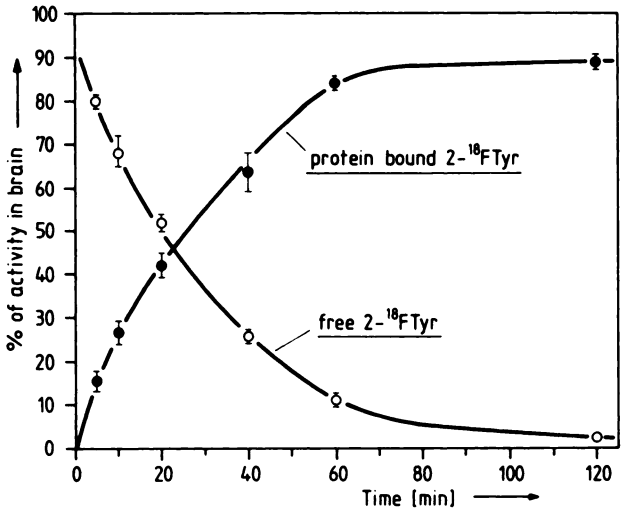


FIGURE 1
Time course of protein bound and free L-[2-^{18}F]fluorotyrosine in cerebral tissue of NMRI mice.

again it could not yet be clearly assigned, for example, to be fluorodopa which is eluted in that region under HPLC conditions employed (*19*). The radioactivity balance of ~97% is almost quantitative in striatal tissue.

Corresponding to a high initial tissue uptake of 2-^{18}FTyr the activity in the blood decreased from a high level of 6.7 ± 0.8% of the injected dose per gram at 5 min p.i. to 1.85 ± 0.3% at 40 min and to 2.1 ± 0.5% at 120 min. Preliminary studies at 20 and 40 min revealed a rather rapid disappearance of free 2-^{18}FTyr from 47 ± 5% to 29 ± 4%, respectively, of the total blood activity. During the same period of time the protein incorporated fraction increases from 11 ± 2% to 35 ± 3%, respectively, of total blood activity. Plasma activities of 7 ± 2% and 22.5 ± 3.6% were found as free fluoride at these times.

DISCUSSION

The most important biochemical property to be tested of an analog tracer for the assessment of protein

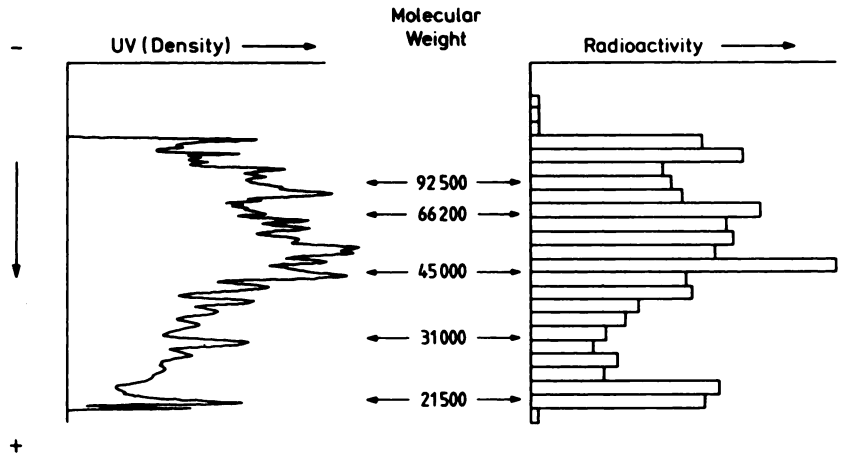


FIGURE 2
Radioactivity and uv-absorbance distribution after molecular mass separation of 2-^{18}FTyr-incorporated cerebral proteins by discontinuous SDS-gel electrophoresis.

synthesis in vivo is its acceptance by the aa-tRNA synthetase and its subsequent incorporation into peptide chains by ligase enzyme systems. The generally applied method of isolating proteins from tissue homogenates by trichloroacetic acid (TCA) precipitation is rather crude. It especially does not discriminate between real incorporation into the peptide chain and possible binding as false substrate to one or several enzymes, with which it would be coprecipitated by TCA. Several halogenated amino acids are known as inhibitors of various enzymes (*15,23*). In addition, the finding of a small fraction of 2-^{18}FTyr of 1.5–2% of tissue activity in the aa-tRNA pool is not sufficient to distinguish between a poor acceptance by the aa-tRNA synthetase or, in contrast, a fast incorporation into proteins.

The separation of TCA-precipitated proteins by their molecular mass by means of SDS gel electrophoresis clearly demonstrated the distribution of ^{18}F activity over the whole molecular range of peptides and proteins (cf. Fig. 2). Coprecipitation of 2-^{18}FTyr must be excluded because of protein denaturation in the SDS electrolyte solution. The binding of 2-^{18}FTyr to some specific enzymes is also disproven because this would result in association of the ^{18}F activity in only one or some distinct molecular mass fractions. The reliability of this analytic method was demonstrated by comparison with L-[3-^{123}I]iodo-α-methyltyrosine, another analog tracer. Here, ~18% of tissue radioactivity was precipitated by TCA in cerebral tissue of mice 40 min p.i. (*14*). However, SDS gel electrophoresis showed migration of the radioactivity exclusively with the monomolecular fraction, thus disproving general protein incorporation of the radioiodinated analog.

The absolute uptake into brain tissue of L-[2-^{18}F]-fluorotyrosine with up to 2% of the injected dose per gram tissue is low, but in the same range as that of other radiolabeled amino acids. It is in agreement with the recent observation that the total net transport of large neutral amino acids into cerebral tissue of rodents is negligible (*24*). This fact also favors fluoroanalogs over [^{11}C]amino acids as there is no endogenous pool of the fluorine tracer in the tissue present. More important than the comparison of tissue uptake is that of protein incorporation data of other radiolabeled amino acids. For example at 40 min postinjection the incorporation into cerebral proteins of rodents of 2-^{18}FTyr with 68 ± 4% is directly comparable to that of the natural analogs L-phenylalanine, L-leucine, L-methionine and L-tyrosine with 63 ± 2% (*17*), 85 ± 4% (*12*), 57 ± 3% (*12*), and 70 ± 4% (*7*), respectively. The metabolism of 2-^{18}FTyr and L-[1-^{14}C]tyrosine (*8*) are surprisingly similar in cortical tissue, suggesting that the introduction of fluorine into the 2-position hardly affects its biochemistry. The protein incorporation of L-[4-^{18}F]fluorophenylalanine as discussed above is much inferior (*14*). 2-Fluorophenylalanine and 3-fluorotyrosine, which are much more easily labeled, are either not accepted for protein synthesis (*17*) or toxic (*15*).

Figure 1 demonstrates the rapid incorporation of 2-^{18}FTyr into cerebral proteins which is ~90% after 2 hr p.i. This, as well as the almost linear increase between 10 and 60 min, corresponds to previously reported protein synthesis rates (*7*). The concentration of the free tracer adds to that of the protein bound fraction to yield an average of 94.5 ± 4.5% of total tissue activity at all times measured. The intermediary aa-tRNA pool from which the protein buildup proceeds undirectionally is small (1.5–2%). This suggests a fast turnover of ^{18}FTyr-tRNA. It is in direct exchange with the free tracer pool and may be combined therewith into one compartment for purposes of kinetic modeling of tissue radioactivity time courses.

The cortex tissue analysis after administration of L-[2-^{18}F]fluorotyrosine thus allows a compartmentalization as shown in Figure 3. In addition to free 2-^{18}FTyr in blood plasma, there are only two measurable fractions which almost quantitatively account for the activity in the tissue when free and tRNA bound activity are combined. The fraction of unidentified metabolites is <3% in cortex tissue. It is on the order of the standard deviations of the free and protein bound fraction of ^{18}F

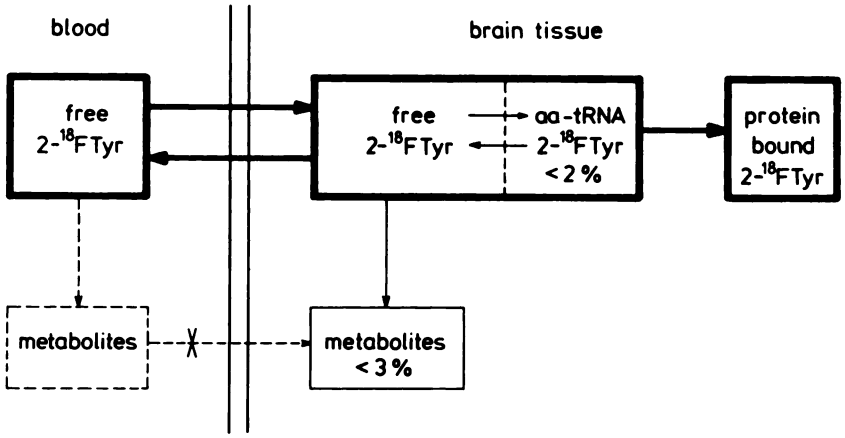


FIGURE 3
Compartment model for metabolism of L-[2-^{18}F]fluorotyrosine in brain tissue.

activity and possibly negligible. Thus, at least in the cortex, the cerebral tissue with highest protein turnover, the physiologic behavior of L-[2-^{18}F]fluorotyrosine can closely be described by a three-compartment model. Such a model, having four rate constants at most, would greatly simplify the quantitation of cerebral protein synthesis rates by PET when compared to existing tracers and models (*3,11,12,17*).

It is surprising that even after 60 min p.i. a significant amount of labeled metabolites was not formed. Judging from the chromatographic behavior of the unidentified compound and its increase in striatum tissue to 4.2%, one might speculate this to be fluorodopa or fluorodopamine. If formed at all, the small fractions indicate that 2-fluorotyrosine is hardly a good substrate for tyrosine hydroxylase in vivo. Because catecholamines originate from the hydroxylation of L-tyrosine (*25*), labeled dopa and dopamine would likely accumulate in the striatum after administration of natural L-[^{11}C]tyrosine. Unfortunately, dopaminergic regions as striatum or substantia nigra were not separately examined in the study with L-[1-^{14}C]tyrosine (*8*). If tyrosine hydroxylation occurs within 1 hr after administration it must be concluded that fluorination in the 2-position of tyrosine apparently inhibits the hydroxylation in the 5-position. It is interesting to note that with the same configuration of F- and HO-substituents in 6-fluorodopa the 3-O-methylation by COMT is drastically reduced when compared to dopa (*26*). On the other hand, 2-fluorophenylalanine apparently is hydroxylated by phenylalanine hydroxylase. All ^{18}F activity in the cerebral pool of aa-tRNA seemed to be in the form of 2-[^{18}F]fluorotyrosine after administration of [2-^{18}F]fluorophenylalanine (*17*). This conversion probably took place in the liver from where the newly formed 2-^{18}FTyr was redistributed by the blood.

After direct administration of L-[2-^{18}F]fluorotyrosine a significant redistribution of metabolites from the body to the brain can be excluded because of the activity balance observed in cerebral tissue. In principle, however, the plasma could be the source of unidentified traces of activity in tissue considering the 30 to 40% of blood activity at 20 and 40 min p.i. that is not bound to tyrosine. Analysis of the blood revealed the expected fast decrease of free tracer concentration in the plasma and a corresponding increase of plasma-protein incorporated 2-^{18}FTyr. A substantial part of plasma activity is free fluoride which is increasing with time. It does not cross the blood-brain barrier but may interfere with measurements in other organs and tumor tissue and will accumulate in bones.

The measurement of the time course of the free tracer in the plasma is necessary to obtain the plasma input function for quantitation of the PSR by the above-mentioned model. This can be easily done by HPLC analysis. Another consideration is the high level of radioactivity in the blood relative to that in cerebral tissue, a fact that necessitates an exact determination of the cerebral blood volume. Furthermore, the active transport into the brain of all large neutral amino acids by one carrier system (*27*) is subject to competition with such amino acids present in the plasma. This was recently demonstrated for L-[6-^{18}F]fluorodopa (*28*) and L-[^{11}C]methionine (*13*) and is a problem for all radiolabeled amino acids. The amounts of 2-FTyr used here (~5 μg) are assumed to be on a tracer level compared to the amount of free tyrosine present in blood. The influence of carrier dose and level of plasma amino acid concentrations, on the other hand, has to be carefully examined particularly in future quantitative PET studies.

CONCLUSION

The data on murine cerebral metabolism and kinetics L-[2-^{18}F]fluorotyrosine strongly recommend this tracer for the evaluation of protein synthesis by PET. The incorporation of 2-^{18}FTyr into cerebral proteins is fast and almost completed within 1 hr. True incorporation can be proven by gel electrophoresis of proteins. In cortical tissue the ^{18}F activity is distributed almost exclusively into two pools, which are the protein bound fraction and free 2-^{18}FTyr. This simple metabolism may allow the application of a three-compartment model for the quantitation of cerebral protein synthesis rates. The possible formation of [^{18}F]fluorodopa and [^{18}F] fluorodopamine in striatum is small even at extended times and therefore its influence on PSR determination is probably negligible. The significance of small traces of metabolites must be examined in further biochemical and dynamic PET studies.

These questions are of even greater importance for tumor tissue, a challenge for diagnostic PET methods based on protein synthesis. A recent comparative multiparameter PET study on cerebral tumours suggest the amino acid-protein metabolism as an important parameter for the evaluation of malignant tissue (*29*). Nothing, so far, is known about the metabolic behavior of L-[2-^{18}F]fluorotyrosine in malignant tissue. However, a preliminary case study together with the Max-Planck Institute for Neurological Research (Cologne, FRG), revealed a clear delineation of a glioblastoma in the brain of a patient (for a PET image see Ref. *30*). Present efforts to optimize the labeling of the tracer by a regioselective fluorodemetallation reaction will facilitate further biochemical tissue studies and extended examinations in patients. These will allow validation of a three-compartment model using L-[2-^{18}F]fluorotyrosine, the evaluation of its clinical relevance, and a comparison with other PET tracers presently used for probing the viability of cerebral and tumor tissues.

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