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Microdialysis as a Tool in Studies of L-Dopa and Metabolites in Malignant Melanoma and Parkinson's Disease

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Abstract

A model with human melanoma xenografts transplanted to athymic mice has been adopted for *in vivo* studies of 5-S-cysteinyldopa (an intermediate pigment metabolite), glutathione, and cysteine. L-Dopa is an intermediate metabolite in pigment formation and is also important in the treatment of Parkinson's disease, and therefore I have also studied the pharmacokinetics of this compound.

We were first to describe *in vivo* microdialysis in melanoma tissue and showed that dialysis membranes of cuprophane or polyamide are suitable for studies of interstitial 5-S-cysteinyldopa and selected thiols. Analytical procedures were also improved for quantitation of 5-S-cysteinyldopa, L-dopa, glutathione, cysteine, and N-acetylcysteine (NAC). In the melanoma xenografts the interstitial concentration of 5-S-cysteinyldopa reflected the high intracellular production of this intermediate metabolite. For *in vivo* manipulation of glutathione in the melanoma tissue we gave intraperitoneal injection of buthionine sulphoximine to the animals and thus reduced the glutathione concentrations substantially. We showed that restitution of glutathione in melanoma tissue occurs spontaneously and is not much improved by treatment with the cysteine deliverers NAC and L-2-oxothiazolidine-4-carboxylate (OTC). 5-S-Cysteinyldopa was not substantially affected by great variations in glutathione concentrations. Transport of NAC from intraperitoneal injection to melanoma tissue occurred rapidly and deacetylation to cysteine *in vivo* could be detected soon after NAC injection. *In vivo* formation of cysteine was slower from OTC than from NAC.

Pharmacokinetic studies of L-dopa in human subjects indicated a slight to moderate protein binding. Plasma free L-dopa had similar elimination $T_{½}$ as interstitial L-dopa, but in some cases the elimination of total L-dopa was slower. Difficulties in intestinal absorption of L-dopa were revealed by microdialysis in blood and subcutaneous tissue. Studies showed that this was due to delayed emptying of the stomach. L-Dopa intake increased 5-S-cysteinyldopa concentrations in blood within 30 min in patients with Parkinson's disease and a history of melanoma. No melanoma activation occurred during long-term treatment with L-dopa.

Microdialysis is thus a safe and easily applied method for *in vivo* studies of both pigment metabolites from human melanoma tissue transplanted to nude mice and for pharmacokinetic studies of L-dopa.

Förord

Vid en första blick på avhandlingens titel kan det tyckas vara omöjligt att kombinera malignt melanom och Parkinson's sjukdom. Men under mitt arbete med att utveckla modeller för att med mikrodialys studera pigmentbildningen och dess metaboliter har avståndet mellan dessa sjukdomstillstånd krympt. Jag hoppas att läsaren finner intresse i mina arbeten, som har kunnat genomföras med hjälp av anslag och stöd från Linköpings Universitet, Svenska Cancerfonden, Neurologiskt Handikappades Riksförbund (NHR) och Östergötlands Läns Landsting.

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Linköping i februari 1999

Nil Dizdar Segrell

Contents

Original papers

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Blomquist L, Dizdar N, Karlsson M, Kågedal B, Ossowicki H, Pettersson A, and Smeds S. Microdialysis of 5-S-cysteinyldopa from interstitial fluid in cutaneous human melanoma transplanted to athymic mice. Melanoma Res 1991; 1: 23-32.

II. Dizdar N, Kågedal B, Smeds S, and Årstrand K. A high-sensitivity fluorometric high-performance liquid chromatographic method for determination of glutathione and other thiols in cultured melanoma cells, microdialysis samples from melanoma tissue, and blood plasma. Melanoma Res 1991; 1: 33-42.

III. Dizdar N, Kullman A, Kågedal B, and Årstrand K. Effects on interstitial glutathione, cysteine and 5-S-cysteinyldopa of buthionine sulphoximine in human melanoma transplants. Melanoma Res 1997; 7: 322-328.

IV. Dizdar N, Kullman A, and Kågedal B. Comparison of N-acetylcysteine and L-2-oxothiazolidine-4-carboxylate as cysteine deliverers and glutathione precursors in human malignant melanoma transplants to mice. Submitted to Cancer Chemother and Pharmacol 1999.

V. Dizdar N, Kullman A, Norlander B, Olsson J-E, and Kågedal B. Human pharmacokinetics of L-dopa studied with microdialysis. Submitted to Clin Chem 1999.

VI. Dizdar N, Granérus A-K, Hannestad U, Kullman A, Ljungdahl Å, Olsson J-E, and Kågedal B. L-Dopa pharmacokinetics studied with microdialysis in patients with Parkinson’s disease and a history of malignant melanoma. Submitted to Acta Neurol Scand 1999.

Abbreviations

AADC	Aromatic L-amino acid decarboxylase
ACTH	Adrenocorticotrophic hormone
BSO	Buthionine sulphoximine
CNS	Central nervous system
CRF	Corticotrophin releasing factor
DACM	N-(7-Dimethylamino-4-methyl-3-coumarinyl) maleimide
GSH	Glutathione (reduced)
GSSG	Glutathione symmetric disulfide (oxidized glutathione)
GSSX	Glutathione, mixed disulfide with another thiol
HPLC	High-performance liquid chromatography
MSH	Melanocyte stimulating hormone
NAC	N-Acetylcysteine
OTC	L-2-Oxothiazolidine-4-carboxylate
TPS	Thiopropyl-sepharose

Introduction

Microdialysis was first described in the sixties and for several years it was used mainly in studies on cerebral transmitters. When we started to use the technique in the beginning of 1990 we had not seen any publications illustrating its use in tumor tissue. Since then the technique has been developed extensively and applied to almost all tissues. It enables measuring of different substances, at steady state conditions and during changes due to treatment, and for short and long periods with minor tissue damage. We developed our model for study of pigment metabolites and we miniaturized our analytical methods to be able to measure the metabolites in very small samples and at low concentrations. We have focused on two conditions, malignant melanoma and Parkinson's disease.

Both melanocytes and nerve cells derive from the neural crest in the embryonic phase. The melanocytes are distributed to the epidermis of the skin, the hair follicles, the leptomeninges, the inner ear, and the eye. The production of melanin pigment is specific for these cells. Dopaminergic nerve cells producing neuromelanin are located in *substantia nigra* of the midbrain. Both these cell types are involved in diseases such as malignant melanoma and Parkinson's disease where pigment metabolites from L-dopa play an important role.

Malignant melanoma is a tumor derived from melanocytes and its incidence rate has been steadily increasing in the white population for the last 30 years. Glutathione is proposed to be an important resistance factor to the treatment of this disease. Different L-dopa metabolites from the pigment formation play an important role in the follow-up of the disease.

Parkinson's disease is due to massive loss of dopaminergic neurons in *substantia nigra*. This loss also causes a shortage of dopamine, which is an important transmitter substance in the midbrain and plays an important role in the synthesis of neuromelanin. An increased understanding of the pigment formation may give us better possibilities in the treatment of the diseases.

Background

Microdialysis

Microdialysis was first developed for intra-cerebral use (1) and is now widely used in different tissues and fluids. The technique is based on passive diffusion of substances across a semi-permeable membrane (2). A probe containing a dialysis membrane is implanted into tissue and perfused with a dialysis fluid at a constant and low flow rate. The measured concentrations of endogenous or exogenous compounds reflect the concentrations in interstitial fluid.

Different microdialysis probes are available but they have all some common components: an inlet tube, a semipermeable membrane, and an outlet tube (Fig. 1). They contain hollow-fiber dialysis membranes made of polycarbonate-ether copolymer, cellulose, cellulose acetate, cuprophane, regenerated cellulose, or polyacrylonitrile with molecular cut-offs of 5-100 kD. Some analytes may stick to membrane surfaces due to the presence of surface charges, resulting in low recovery (3, 4). Studies have shown varying extraction of substances across different membranes (3).

Microdialysis requires sensitive analytical methods to measure low concentrations in small sample volumes and is suitable for on site studies and also for pharmacokinetic and pharmacodynamic studies. It allows studies of complex interactions of drugs at their sites of action in intact living tissue, and leaves endogenous metabolic and oxygenation pathways, as well as synaptic functions, largely intact (5). One advantage of microdialysis in the blood circulation vs. ordinary venous blood sampling is that the sample is protein-free and needs no clean-up, and only the free fraction of the compound of interest is monitored. If the analyte is chemically unstable it can be stabilized directly when collected into the sampling tube. Numerous factors influence tissue penetration of drugs, and monitoring of drug concentrations at their effect compartments therefore allows a more detailed and relevant pharmacokinetic and pharmacodynamic model. Microdialysis also allows continuous registration for longer periods e.g. hours or days, and the samples can be collected in small fractions without blood loss or marked inconvenience for the patient. Limitations of the technique are relatively low recoveries of substances with high molecular weight or highly lipophilic compounds, and the technique require sensitive analytic methods (6, 7).

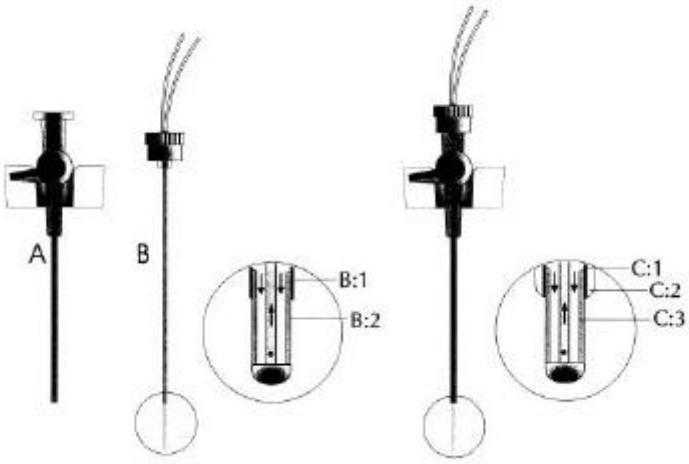

Fig. 1 Microdialysis probe for intravenous use developed by CMA Microdialysis AB in accordance with our wishes. Venflon® catheter (A) and the intravenous probe (B) separate and assembled (C). The attachment of the dialysis membrane (B: 2, C: 3) to the probe (B: 1, C: 1) is positioned inside the Venflon® catheter (C: 2).

The dialysis probe is placed in the tissue to be investigated and is in close contact with the interstitial fluid and the cells of the tissue. The concentrations monitored thus reflect the concentrations of the extracellular fluid. In our studies we have tried to illustrate how this compartment is in dynamic interchange with the intracellular content and with the blood circulation.

Recovery

The dialysis properties of a probe are described in terms of recovery of a particular substance, which is continuously removed from the site of action by diffusion across the probe membrane. The concentration of the analyte collected in the perfusion fluid does not reach true equilibrium with the extracellular concentration (8, 9). However,

under constant experimental conditions a nearly steady-state recovery will be reached quickly. This relative recovery (10) represents a constant fraction of the extracellular concentration and it can be determined from *in vitro* recovery experiments. It is calculated by dividing the concentration of the substance in the dialysate samples by the concentration in the sampled medium. Since the process depends on simple diffusion, the rate of transfer across the membrane is equivalent at all concentrations under otherwise constant experimental conditions. Relative recovery increases with increasing length of the probe and with decreasing flow rate of the dialysate fluid. Absolute recovery (11, 12) represents the total amount of substance collected per unit time and increases with increasing flow rate of the dialysis fluid.

It is necessary to measure the *in vitro* recovery to be able to evaluate if the recovery is adequate for sampling of a particular analyte, to choose an optimum flow rate, to determine probe-to-probe variability, and to evaluate probe integrity after experiments.

The recovery obtained in an experimental situation is proportional to the diffusion coefficient for the particular substance in the probe membrane matrix. Further, the whole diffusion process is also influenced by other characteristics such as diffusion and convection phenomena occurring in the tissue, tissue tortuosity and volume fraction, microvascular transport, and active elimination of an analyte through metabolism or uptake into cells.

In vivo recovery has been used in attempts to reflect the true extracellular concentration of the compound measured. Two methods to determine *in vivo* probe recovery under steady-state conditions are the “no net flux” (13-15) and the “zero flow” methods (16). In the first one the probe is perfused with different concentrations of the analyte of interest. The extracellular concentration is determined by the point at which no net gain or loss occurs from the perfusion fluid as measured in the dialysis efflux. In the zero flow method, the dialysate concentration is measured at various perfusion flow rates, plotted against flow rate and extrapolated to zero. The concentration at zero is said to equal the extracellular concentration.

Reverse dialysis (retrodialysis) (10, 17) can be used for recovery estimation *in vivo* during non steady-state conditions. One advantage of retrodialysis is that it is reproducible in a particular tissue. This technique assumes that the diffusion properties are equal from either side of the dialysis membrane. The probe is perfused with a solution containing a known concentration of the analyte of interest and the percentage

loss of the analyte from the perfusate is measured in the dialysate samples. The loss of the substance from the perfusate is a measure of the efficiency of movement of the analyte across the membrane under defined conditions in a specific tissue. Internal standard methods (18, 19) require addition of a substance, which optimally mimics the diffusion properties of the analyte, to the perfusion fluid. One of the disadvantages with this method is that competition or interference of the internal standard with the distribution, metabolism or elimination of the analyte can occur.

It has been shown that *in vivo* recovery data from probes implanted in biological fluids such as blood or bile are in good agreement with *in vitro* recovery experiments (20-22).

Pigment formation

Melanin pigments are classified into two main groups: brown - black eumelanin and yellow to reddish brown pheomelanin. The synthesis of the pigment occurs in the melanosomes of the melanocytes (23, 24). The melanin pigment is formed in a similar way in the melanoma cells.

Melanin formation starts from the amino acid tyrosine (Fig. 2). By the action of the rate-limiting enzyme tyrosinase, L-dopa and subsequently dopaquinone are formed. In the absence of sulfur-containing compounds the indolic pathway follows, with cyclization of dopaquinone and formation of cyclodopa, which after oxidation gives dopachrome. Dopachrome is then either spontaneously decarboxylated to 5,6-dihydoxyindole (DHI) or converted to 5,6-dihydoxyindole-2-carboxylic acid (DHICA) by the action of the enzyme dopachrome tautomerase. DHI and DHICA are further oxidized to 5,6-indolequinone and 5,6-indolequinone-2-carboxylic acid respectively. After polymerisation of the indolequinones the black eumelanin is finally formed (25, 26).

If glutathione or cysteine is available nucleophilic addition to dopaquinone occurs and cysteinyldopas or glutathionyldopas are formed (27). In the latter case, by the action of the enzyme γ-glutamyltranspeptidase the glutathionyldopa is converted to cysteinyldopa, which undergoes oxidative cyclization to 4-benzothiazines. After polymerization of the benzothiazines the red pheomelanin is obtained.

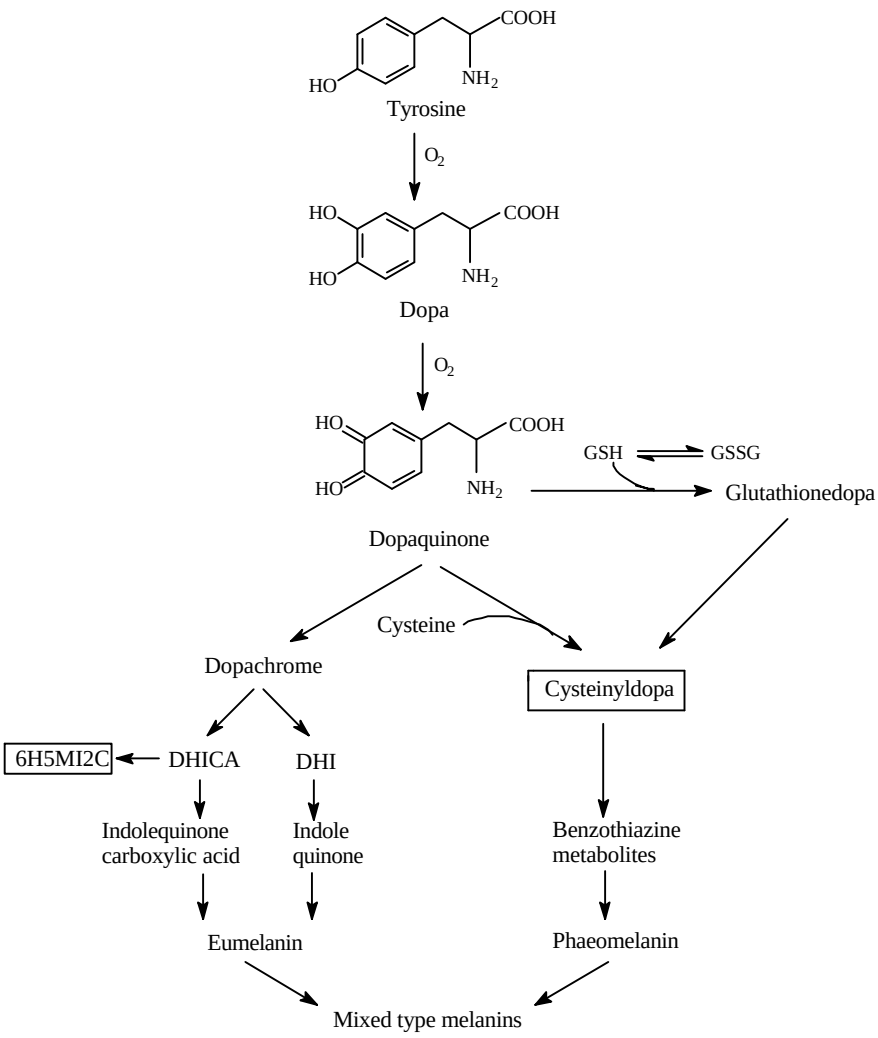

Fig. 2 Pigment formation in melanocytes and melanoma cells.

Malignant melanoma

The primary etiology of melanoma is unknown but there is strong evidence that UV-light exposure plays a major role in the development of the cutaneous melanoma (28, 29). The incidence of this disease has increased markedly during the last three decades in the Caucasian population all over the world (30, 31). It has been shown that the increase in incidence is real and not due to improved diagnostic skills, better methods of counting cases, or change of histologic criteria (30). At various locations of the melanocytes neoplastic transformation to malignant melanoma generates different types of melanomas, namely cutaneous melanoma, uveal melanoma, and melanoma of the mucous membranes.

There are five types of cutaneous malignant melanoma: a) *In situ* malignant melanoma, which is the pre-invasive stage of melanoma, localized to the epidermis.

The tumor is entirely flat or appears as a small very dark macule. b) Superficial spreading malignant melanoma is the most common type (over 50 %). The tumor presents as a slightly raised lesion with irregular margins and variable pigmentation. c) Nodular melanoma invades vertically from the start and has a worse prognosis. d) Acral lentiginous malignant melanoma, which is rare in Caucasians, occurs on the palms, soles, and mucous membranes. e) Lentigo maligna melanoma is histologically similar to the acral lentiginous type but has better prognosis. It occurs on the face especially in the elderly and is associated with chronic sun exposure.

To determine the prognosis of the lesions the Clark levels (stage I-IV) are often used (32). The most important prognostic factor in primary melanoma (stages I and II) is the tumor thickness (33), which influences the risk for local recurrence and metastasis. It has also been suggested that the tumor depth can be a prognostic factor (34). Also ulceration is an important prognostic factor, which is strongly associated with tumor thickness (35).

Cutaneous malignant melanoma metastasizes to the regional lymph nodes through the lymphatics and by hematogenous spread mainly to the lungs, liver and central nervous system (36). The number of nodal involvement in stage III melanoma is an important prognostic indicator. Distant metastatic melanoma (stage IV) has poor prognosis. The mean duration of survival for those patients is 6-18 months after progression of the disease (37).

Animal models with nude mice were developed for study of the properties of human tumors including malignant melanoma at the end of the 1960-ies (38). It was described that tumors produced in animals after heterotransplantation of human malignant melanoma cell lines to nude mice were histologically similar to those in humans (39). Later various experiments have been performed studying the effects of different therapies on melanoma xenografts or on metastases (40, 41).

It is well known that malignant melanoma is therapy resistant and also contains high concentrations of glutathione, which has protective functions against damage by toxic compounds, reactive oxygen intermediates and irradiation (42-44).

Glutathione

Glutathione is a tripeptide, which is present at high concentrations in almost all living cells. It is synthesized by the action of the enzymes γ-glutamyl cysteine synthetase and glutathione synthetase from glutamate, cysteine and glycine (45). Intracellularly it is present in the reduced form (GSH, 95 %), as disulfide (GSSG, 3 %) or conjugated to proteins (GSSX, 2 %) (46).

Glutathione takes part of a number of important cellular processes. One of its functions is to detoxify reactive intermediates formed intracellularly either spontaneously or enzymatically (44, 47). The conjugation is catalyzed by glutathione S-transferases, which also function as binding proteins (48). Glutathione is also considered to functions as a storage form for cysteine (49-52).

The relation between the intracellular concentrations of glutathione and cellular resistance to toxic agents has interested several investigators. The modulation of glutathione levels in cell cultures and liver has been studied (53-56). It has been shown that decrease of glutathione levels can be achieved by treatment with buthionine sulphoximine (BSO), which is a very potent inhibitor of the enzyme -glutamyl cysteine synthetase (54, 57). The formation of glutathione can on the other hand be increased by administration of L-2-oxothiazolidine-4-carboxylate (OTC) and N-acetylcysteine (NAC), which are metabolized to cysteine (55).

Parkinson's disease

Parkinson's disease occurs in all cultures and is a common neurologic disease. The incidence in Sweden is 11 per 100,000 inhabitants per year (58). The cardinal clinical symptoms are rigidity, tremor and bradykinesia, of which two signs must be present for diagnosis. The symptoms occur when approximately 80 % of the pigmented dopaminergic neurons of the *substantia nigra* are lost (59).

With the loss of nerve cells there is a shortage of dopamine in the central nervous system (CNS) causing the symptoms of the disease. Treatment is mainly with L-dopa since dopamine does not pass the blood-brain-barrier. L-Dopa is absorbed into the blood from the small intestine (60) and it is transported across the gut endothelium via an enzyme that also transports neutral amino acids. Studies have shown that parkinsonian patients have delayed gastric emptying, which could affect the medication (61). In blood L-dopa is then metabolized by the enzyme aromatic L-

amino acid decarboxylase (AADC) (62). L-Dopa is therefore usually given together with a systemic dopadecarboxylase inhibitor (carbidopa or benserazide) to give a larger fraction of L-dopa to be converted to dopamine in the CNS. An enzyme system mediates the transport across the blood-brain barrier and in the presence of high plasma amino acid concentrations the transport of L-dopa can be reduced (63). In the CNS L-dopa is metabolized by the enzyme AADC into dopamine.

The pigment formation in the nerve cell is regulated by the rate-limiting enzyme tyrosine hydroxylase (64). Similar to tyrosinase in the melanocyte it converts tyrosine to L-dopa. L-Dopa is then by the action of AADC converted to dopamine, which is the main transmitter substance of the dopaminergic neurons (Fig. 2). The neuromelanin is then generated through auto-oxidation of dopamine (65, 66). It has been shown that the neuromelanin, similar to the melanin of the melanocytes, probably consists of mixed-type melanin (67).

Aims

The aims of this work was to increase our knowledge of the pigment formation, to give us a tool to improve the diagnosis and follow-up of patients with malignant melanoma, and also to improve the medical treatment of patients with Parkinson' disease.

The aims of the separate papers were:

1. To develop a model for studies of interstitial 5-S-cysteinyldopa in melanoma tissue.

2. To develop miniaturized methods for analysis of glutathione and other thiols suitable for analysis of these compounds in very small biological samples.

3. To study the role of glutathione and cysteine in the formation of 5-S-cysteinyldopa in human melanoma transplants *in vivo*. Also to study the modulation of glutathione concentrations in melanoma interstitial fluid by BSO, OTC and NAC.

4. To study the long-term effect of BSO, OTC and NAC on the concentrations of glutathione, cysteine, and 5-S-cysteinyldopa in melanoma tissue. To investigate if the interstitial fluid concentrations reflect the intracellular conditions.

5. To develop a procedure for intravenous use of microdialysis sampling in patients and to study the pharmacokinetics of L-dopa in blood and tissue. To investigate the L-dopa balance between blood circulation and interstitial fluid.

6. To study the relation between the concentrations of L-dopa and 5-S-cysteinyldopa in patients with a history of both Parkinson's disease and malignant melanoma.

Material and Methods

Animals

Male athymic mice (nu/nu – Balb/cABom), 25 mg of weight, were used in all animal experiments. About 10^7 melanoma cells from cell culture were injected at four locations subcutaneously on the back of the mice. The tumors were about 10 x 10 x 5 mm when used. The mean total tumor burden was 1.7 % of body weight. None of the animals had any metastases at the autopsy performed at the end of the experiments.

For general anesthesia of the mice during the microdialysis 0.1 mL of xylazine (Rompun® 20 mg/mL) and 0.4 mL of ketamine (Ketalar® 50 mg/mL) was mixed with 0.5 mL of sodium chloride (154 mmol/L), and 0.12 mL of the mixture was given as intraperitoneal injection. The mice were then placed on a temperature controlled (35–36 °C) electric pad. Maintenance of anesthesia was with subcutaneous injection of 0.05 mL of the mixture every 30-min.

Patients

In paper V the patients with Parkinson's disease were in early stages of the disease since our primary interest was to develop and test our human model of sampling. Ten patients aged 44-68 years participated in the study. Mean duration of the disease was 3.2 (± 1.2) years and mean duration of L-dopa treatment was 1.6 (± 0.8) years. The daily L-dopa dose was 340 (± 70) mg.

In the sixth paper five patients with Parkinson's disease and with a history of malignant melanoma were studied. They were 55-78 years of age, and the mean duration of their disease was 6.2 (± 4.6) years. The daily dose of L-dopa was 570 (± 452) mg and the mean duration of treatment was 4 (± 5.2) years.

Nine healthy volunteers were studied in paper V. They had no history of movement disorders or any medication. The mean age was 51 (± 11) years with the range 40-69 years. *In vivo* recovery of L-dopa was performed in two healthy subjects 26 and 27 years of age. In paper VI fifty healthy volunteers were used to obtain a normal range for the gastric half emptying time with the [^{13}C]-octanoic acid breath test. The mean age of these subjects was 44 (± 9.0) years with a range of 24-59 years.

Cell culture

We used cultured melanoma cells from different lines. The cell lines used were IGR 1 obtained from Dr. C. Aubert (Marseille, France), JKM 86 – 4, M5, and M9 from Dr. U. Stierner (Gothenburg, Sweden), Mel 28 and B16F1 from ATCC (USA).

Cultures were performed in different media all described in the papers. The melanoma cells were cultured in flasks (culture area 80 cm^2) or in multi-well plates (9.6 cm^2) from A/S Nunc (Roskilde, Denmark). We used 12 or 20 mL medium and added 1-2 mL of primary culture (1-3 x 10^6 melanoma cells) for the culture flasks. Seeding in multiple wells was with 1-8 drops of cell suspension (0.5-1.5 x 10^6 melanoma cells) to 3 mL of medium in each well. Culture was performed for either 6-8 days in flasks or for 1-2 days in multiple wells at 37 °C and 5 % CO_2 in an ASSAB CO_2 incubator.

Cells for thiol analysis were prepared as follows. The culture medium was decanted from the flasks or soaked off from the multiple-well plates. The cells were washed once with phosphate-buffered saline (PBS) and then to the flasks, 6 mL of trypsin (0.25 %) was added and to wells, 1 mL was added. Incubation was continued for 1 min at room temperature. After decanting, incubation was continued for another 5 min at 37 °C and the cells were then suspended in culture medium or PBS, 5 mL to flasks and 0.5 mL to multiple-well plates.

Microdialysis

We used a microdialysis set from CMA Microdialysis AB (Stockholm, Sweden) which consisted of a CMA/100 microinjection pump with 1-mL syringes connected to the probes. In the first human study (paper V) both the subcutaneous and the intravenous probes were connected to syringes placed in the CMA/100 microinjection pump with a perfusion flow rate of 1 mL/min. In the sixth paper the subcutaneous probe was connected to a syringe placed in a CMA/106 microinjection pump with a perfusion flow rate of 0.3 mL/min.

In animal experiments we used the microdialysis probes CMA/10 and CMA/11. In human experiments CMA/60 was used subcutaneously and a newly constructed probe was used in the blood circulation. The membranes of the microdialysis probes used consisted of polycarbonate, cuprophane and polyamide with a molecular cut-off at 20,000 D. The

dialysate solution used was Ringer acetate. In the human experiments we used 20 IU/mL of sodium dalteparin (Fragmin®) in the dialysate solution to inhibit the formation of fibrin deposits on the membrane.

[^{13}C]-Octanoic acid breath test

Gastric emptying was measured by the method described by Ghoos et al. (68). After at least 12 hours fasting the subjects came to the Department of Clinical Chemistry and the test was started with collection of two breath samples. A test meal was prepared. The energy content of the meal was approximately 250 kcal, proteins contributing with 15 %, fat with 62 % and carbohydrates with 23 %.

The patient was told to ingest the test meal in less than ten minutes and to drink 150 mL of water immediately after the meal. Breath samples were then collected every 15th min for 4 hours with the subject seated. All samples were collected in 10-mL tubes. The breath samples were analyzed by continuous flow isotope ratio mass spectrometry.

To establish the normal range of the gastric half-emptying time fifty healthy volunteers (25 men and 25 women) were recruited. The mean age was 44 years with a range of 24 – 59 years. None of them had a history of upper gastrointestinal surgery or was on medication affecting the gastric emptying time.

Analytical method

Analysis of 5-S-cysteinyldopa

We used earlier described methods for analyses of 5-S-cysteinyldopa in urine and serum (69-71). For analyses of very low concentration we also used a modified version of the above mentioned methods. The mobile phase contained phosphate buffer, 0.1 mol/L, and sodium lauryl sulfate, 3.5 mmol/L, to which acetonitrile was added to a final concentration of 15 %. Without pretreatment 5 µL of dialysate was introduced into the chromatograph by loop injection.

Analysis of glutathione

A direct method for GSH and GSH + GSSG with DACM derivatization was developed. The method is fully described in paper II. The method

allows determination of glutathione in 10 µL samples at low concentration with quantitative recovery. Limit of detection was 5-10 fmol/ sample (paper II).

Analysis of L-dopa

L-dopa was analyzed by HPLC with electrochemical detection. The clean-up of serum samples is described in paper V. The microdialysates were injected into the HPLC-system without further clean-up.

Statistics

Results are expressed as mean ± standard deviation (SD). The data were analyzed by student's *t*-test (paired differences). $P<0.05$ was considered to be significant.

Pharmacokinetic calculations were done using Excel 5.0 working under Windows 3.11. The areas under the different concentration-vs-time curves up to the last sample at 240 minutes were estimated by the trapezoid rule. After visual assessment of the semi-logarithmic plot of the same curves the pharmacokinetic parameters (intercept concentration at zero time for the dose (C_0), elimination half-life and time to peak) were calculated with the same program.

Mann-Whitney U-test was used to set the normal range of gastric half-emptying time.

Results and Discussion

Microdialysis in melanoma tissue

Technical considerations

A number of problems are associated with the microdialysis technique. Thus, variable results may be due to instability of compounds, variable tissue and plasma protein binding, and adsorption of the compound to the dialysis membrane or tubing. The membrane itself may also affect the diffusion of the substance of interest even if the molecular size is far less than the cut-off of the membrane. Release of the compound from the cells and diffusion rates have to be taken into account in quantitative analysis. In paper I we experienced these difficulties and found that 5-S-cysteinyldopa, which was the compound of interest, seemed unstable at very low concentrations and at neutral pH. Experiments with different antioxidants improved the stability of 5-S-cysteinyldopa (paper I, Table 1). We also had low recovery figures of 5-S-cysteinyldopa in serum with the polycarbonate membrane, both with a cut-off of 20 kD (36 %) and with 100 kD (7 %). Ultrafiltration of serum spiked with 5-S-cysteinyl[^{3}H]L-dopa showed that the low recoveries could not be explained by protein binding. Comparing of three different membranes (polycarbonate, polyamide and cuprophane) showed higher recovery with polyamide and cuprophane membranes indicating that the diffusion *per se* across the membrane can be a cause of low recoveries.

Although a number of factors have to be taken into consideration, the microdialysis in human melanoma tissue transplanted to athymic mice is easily performed and reproducible. The probe is easily introduced into the tumor tissue without causing large tissue damage, and the membrane is placed close to the membranes of intact tumor cells (Fig. 5 in paper I). In the first samples collected after insertion of the probe high concentrations of the substance of interest were seen probably due to cell damage with leakage of the intracellular contents to the extracellular fluid. Intracellular glutathione concentration in melanomas varied widely (paper II and III). In the cell line (JKM 86-4) used in paper III we found that the levels varied from 7.6 to 20.0 fmol/cell when they were harvested at different times after seeding. The mean intracellular concentrations of glutathione, cysteine and 5-S-cysteinyldopa are, 24.1 fmol/cell, 0.95 fmol/cell and

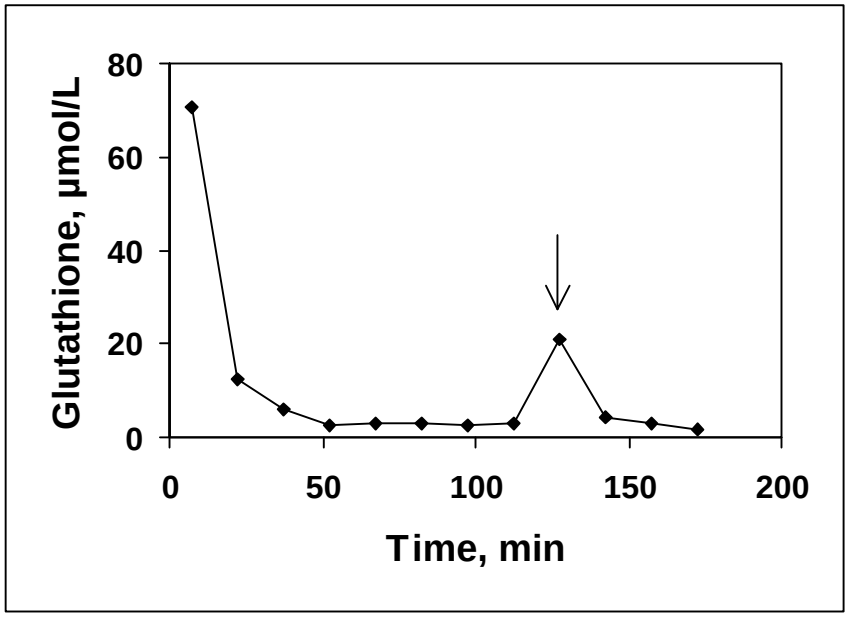

Fig. 3 Microdialysate concentrations of GSH reaching steady-state level after 30 min. Fluctuation of GSH concentration due to change in position of the dialysis probe (arrow).

0.26-1.2 µmol/g melanoma tissue, respectively. Thus the concentrations are well above the microdialysate concentrations obtained in the initial phase, which would reflect initial leakage from the cells. Steady state is reached after 30-45 min but is easily disturbed when the position of the probe is changed (Fig. 3). The steady-state concentrations reflect the extra-cellular concentrations of the substances. Larger mass transfers can be obtained with high flow rates of the perfusion fluid. In our studies we chose the relatively low flow rate of 1 µL/min since we primary were interested in reaching equilibrium with the extracellular fluid. The concentrations obtained of the compounds were sufficient for measurements by our methods also after dilution.

Microdialysis of 5-S-cysteinyldopa in vitro

In our first experiments (paper I) we found it necessary first to perform *in vitro* studies to be able to choose a probe with adequate recovery for sampling of 5-S-cysteinyldopa and to choose an optimum flow rate.

Microdialysis sampling from both serum samples and Ringer-acetate solutions showed that the permeability of the polycarbonate membrane (cut-off 20 kD) increased markedly at low pH compared to neutral pH. The recovery did not improve when we used a membrane with a cut-off

of 100 kD. The recovery of 5-S-cysteinyldopa added to Ringer-acetate increased to 64 % at pH 4.0 compared to 36 % at pH 7.0. The corresponding figures for 5-S-cysteinyldopa added to serum were 56 % and 11 % respectively. We also noted that 5-S-cysteinyldopa was not stable at low concentrations even at the low level of pH 3.0. Addition of Na_2EDTA improved stability and best recovery was obtained with thioglycolic acid, 3 mmol/L and Na_2EDTA, 0.1 mmol/L.

Because of the low recovery of 5-S-cysteinyldopa in serum we investigated the protein binding of the compound. We could show that 80 % of radioactively labeled 5-S-cysteinyldopa was freely dialyzed through the 30 kD membrane used. The experiments showed that neither instability of 5-S-cysteinyldopa nor protein binding could be the cause of low recoveries from serum. We therefore tested three different dialysis membranes and found that recoveries were higher with cuprophane and polyamide than with polycarbonate membranes.

In further experiments with cuprophane membranes slightly higher recoveries were obtained from serum at pH 4.0 compared with pH 7.0. The recoveries from water solutions continued to be higher compared with those from serum. The stability of 5-S-cysteinyldopa was also satisfactory and the use of antioxidant and chelator did not affect the results markedly.

The differences found in the recovery studies from *in vitro* experiments were also mirrored in the results obtained *in vivo* showing the necessity of thorough pilot experiments before studying new analytes with microdialysis.

Analytical procedures for cysteinyldopa and glutathione

Catecholamines are often purified from urine by alumina adsorption at alkaline pH followed by desorption with a strong mineral acid (72, 73). Addition of a strong cation exchanger in the clean-up improves the purification. However, 5-S-cysteinyldopa is unstable at high pH and the recovery is therefore low with this method. With immobilized boronate the affinity for the various catecholamines depends on the pH, and by decreasing the pH of the eluent the catecholamines can be eluted separately. By avoiding pH higher than 5.6 in the prepurification procedure of 5-S-cysteinyldopa from urine the recovery is improved (70). In our first series of experiments on melanoma tissue utilizing polycarbonate membrane (paper I) we obtained very low microdialysate concentrations of 5-S-cysteinyldopa and we therefore had to modify our earlier HPLC procedure.

However, with microdialysis membranes of cuprophane the concentrations were markedly higher and we could use our previous procedure.

In paper II a sensitive and specific method for glutathione is described. Earlier methods were not suitable for the analysis of microdialysis samples. A number of derivatization reagents can be used for the determination of thiol compounds (74) and one of the most sensitive reagents for this is the fluorogenic compound N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM) (75). Derivatization of glutathione with DACM was therefore performed and the derivative was found to be stable at room temperature. Reduction was performed with glutathione reductase to include the symmetric glutathione disulfide in the quantitation. In the chromatogram two main peaks were formed and with increasing amount of acetonitrile in the mobile phase the glutathione derivatives were fused into one peak. Standard curves were linear within the investigated range of 0.34-6.7 pmol per injection.

Cysteinyldopa in melanoma tissue

In the first series of experiments (paper I), microdialysis was applied for the first time to measure 5-S-cysteinyldopa in human melanoma. Ten animals were used and three probes with polycarbonate membranes were inserted, one in each tumor. Microdialysis was performed and the dialysate was collected in phosphate buffer 0.3 mol/L, pH 3.0. In eight of ten animals no 5-S-cysteinyldopa could be found but the remaining two had concentrations of 100 nmol/L and 4 nmol/L in the dialysates respectively. These results confirmed our finding from the *in vitro* experiments.

The recoveries were improved also *in vivo* when the experiments were repeated with cuprophane probes. This time high dialysate concentrations of 5-S-cysteinyldopa were found in ten out of ten animals. We found concentrations of around 2 µmol/L in the initial fractions and about 0.3 µmol/L at steady state. For the first time, we could show that microdialysis was easily applied in studies of 5-S-cysteinyldopa in human melanoma tissue.

The effect of BSO, which is a potent inhibitor of the rate-limiting enzyme γ-glutamyl-cysteine synthetase in the synthesis of glutathione was studied in paper III. We showed decreased concentrations of glutathione in human melanoma and thus confirmed the results from studies on melanoma cell cultures and liver tissue (76-79)]. In the initial dialysate fractions from human melanoma transplants 5-S-cysteinyldopa concentrations decreased significantly to 3.4 - 1.7 µmol/L during BSO treatment

(paper III and IV). We also found increased 5-S-cysteinyldopa concentrations of 0.16 - 0.26 µmol/L in the dialysates from steady-state fractions.

In a second series of experiments in paper III a single dose of NAC, 2 mmol/kg or OTC, 2 mmol/kg were given to animals pretreated with BSO. Thus when cysteine was supplemented with high concentrations of NAC or OTC no increase of 5-S-cysteinyldopa was seen, suggesting that cysteine is not a limiting factor for 5-S-cysteinyldopa synthesis. In paper IV we studied the effect of prolonged treatment with NAC or OTC. The animals were pretreated with BSO, 3 mmol/kg. Then subgroups were concomitantly treated with NAC, 2 mmol/kg or OTC, 2 mmol/kg for 3 days. We could show an acute increase of cysteine and NAC in the melanoma tissue in both paper III and IV but we did not obtain any persistent increase of cysteine levels. On the contrary, we saw further decrease of both cysteine and glutathione during this stage of treatment.

Decreased glutathione levels were observed by Karg et al. (77) when melanoma cell cultures were incubated with BSO or cysteine. Our results also suggest that concomitant treatment with cysteine and BSO potentiates the depletion of glutathione. Benathan found increased concentrations of cysteine and 5-S-cysteinyldopa after BSO treatment of melanoma cells in culture (80). Addition of L-dopa enhanced the 5-S-cysteinyldopa synthesis. It is suggested that 5-S-CD is formed in human melanoma cells by a tyrosinase-dependent mechanism involving the addition of cysteine to dopaquinone and that GSH is not directly implicated in 5-S-CD formation, but that it regulates cysteine levels via the enzyme γ-glutamylcysteine synthetase. Our results with increased interstitial 5-S-cysteinyldopa concentrations during BSO treatment corroborate those of Benathan. The decrease of cysteine during BSO treatment could be due to the consumption of cysteine for the formation of 5-S-cysteinyldopa. Also other authors suggested that 5-S-cysteinyldopa is formed in the presence of thiols in melanocytes (81). We could not show any acute increase of interstitial 5-S-cysteinyldopa after cysteine substitution although we did show increased cysteine concentrations within 30 min after NAC injection.

Glutathione in melanoma cells and tissue

In our studies from paper II reduced glutathione was identified in extracts from melanoma cells and the chromatograms showed very little content of other free thiols. When a reduction step with glutathione reductase or thiopropyl-sepharose (TPS) was included in the procedure only a small increase of glutathione was obtained. From these experiments we

concluded that the main content of the glutathione was in the reduced form and there was a low content of other free thiols in the melanoma cells. The method was sensitive enough to analyze glutathione from cell extracts and we observed a wide range in glutathione concentrations in different melanoma cell populations. High levels of cysteine but no glutathione were found in the incubation media. The considerable variability in glutathione content of melanoma cells was also confirmed by Coates et al. (82).

In paper II we also added melanocyte-stimulating hormone (MSH, 5.0 µg/mL), adrenocorticotrophic hormone (ACTH, 10 µg/mL) and corticotrophin releasing factor (CRF, 10µg/mL) to the incubation media of subgroups of melanoma cell cultures. No significant change of the glutathione levels was observed after treatment with these hormones.

Melanoma transplants were obtained by injection of melanoma cells to the back of athymic mice. The transplants were excised and homogenized and the intracellular content of reduced glutathione was analyzed. It was 2.9 pmol/mg tissue, which was large in relation to the small amount of cysteine 0.07 pmol/mg tumor tissue (paper I).

Microdialysis was performed *in vivo* in tumor transplants on mice (paper II) for 45 min and the dialysates were then pooled. For the first time interstitial thiol concentrations from melanoma tissue, obtained by *in vivo* microdialysis, were reported. Glutathione concentrations were 118 and 102 µmol/L for total glutathione (GSH + GSSG) and GSH respectively and the free cysteine concentration was 8.6 µmol/L. After correction for recovery the extra-cellular concentrations were calculated to be 243 and 204 µmol/L for total and reduced glutathione and 12 µmol/L for cysteine. At this time we used a dialysis membrane of polycarbonate with lower recovery values than in our following experiments. The results thus may be moderately underestimated.

Plasma unbound concentrations of glutathione were also measured. The glutathione content in plasma was mainly in reduced form at a concentration of 2.3 µmol/L. The total glutathione content was 3.0 µmol/L and the cysteine level was found to be 2.7 µmol/L.

It is known that glutathione has an important role in pigment formation but also as a resistance factor in melanoma to radio- and chemotherapy. The mechanism is not clear and we wanted to study the relations between glutathione, cysteine and 5-S-cysteinyldopa by modifying the glutathione concentrations. In paper III we therefore tried to modulate the glutathione concentrations in the extracellular fluid. Buthionine sulphoximine (BSO) suppresses the formation of glutathione by inhibition of the action of the enzyme γ-glutamylcysteine synthetase. At the steady state level reached

after 60 min we could show pretreatment levels of extracellular glutathione of 5.84 ± 2.28, with microdialysis. Mice with human melanoma transplants were then treated for 7 days with BSO, 3 mmol/kg by intraperitoneal injection twice a day. The microdialysis was repeated on the 3rd and 7th day of BSO treatment. The mean glutathione concentrations decreased drastically ($p<0.0001$) during the BSO treatment, independently of time of sampling during the dialysis. The glutathione levels recovered spontaneously after cessation of BSO. We also studied the possibility to break the inhibition of BSO by providing high concentrations of cysteine by intraperitoneal injections of N-acetylcysteine or oxothiazolidine carboxylate (OTC). However, we did not see any recovery of the glutathione levels after these injections. Cysteine, on the other hand, increased significantly after the N-acetylcysteine injection.

Cysteine

It was earlier shown that NAC and OTC are good cysteine deliverers and increase the glutathione levels in the liver (56). In paper III and IV we studied the effects of both acute and chronic treatments with these compounds. Cysteine itself is toxic for the central nervous system and is therefore not suitable for administration on animals and human beings (83, 84).

During continuous microdialysis we found a significant increase of cysteine in the dialysates within 30 min after NAC injection. This illustrates the possibilities of using the microdialysis technique in studies of dynamic changes. After OTC injection no significant increase of cysteine was observed in the dialysates. This could be explained by the fact that cysteine is formed in a two-step reaction from OTC (55) resulting in a prolonged time for cysteine formation.

In the second series of experiments we gave repeated treatment of NAC or OTC to subgroups of animals. After initial depletion of glutathione with BSO, we continued with a combined treatment of BSO and NAC or OTC during 3 days to study the possibility of breaking the inhibition of γ-glutamyl-cysteine synthetase by BSO. However, we could not observe any increase of glutathione or cysteine during this period. On the contrary we observed a decrease of both compounds. Finally the animals were treated with either NAC or OTC after cessation of BSO. After this period we saw a recovery of both glutathione and cysteine although it was not larger than the spontaneous restitution seen in paper III. This strongly

supports the idea that there is a rather unlimited amount of cysteine available for the cell.

Microdialysis in pharmacokinetics

In accordance with our wishes CMA Microdialysis AB developed a microdialysis probe for intravenous use (Fig. 1). The probe was easy to introduce into a Venflon® catheter, already inserted into the vein. In our first experiments the L-dopa concentrations were unexpectedly fluctuating. However, in some of the pilot experiments we noted a fibrin layer on the dialysis membrane. After addition of low molecular heparin to the dialysis fluid no unexpected variation of the L-dopa levels have been seen.

Microdialysis of L-dopa in human subjects

The pharmacokinetics of L-dopa in blood and tissue were studied in paper V and VI using microdialysis. We compared L-dopa concentrations from ordinary serum samples with those obtained from intravenous and subcutaneous dialysis. In paper V we found good accordance between the dialysates from blood and subcutaneous tissue. Only a slight tendency to delay in mean time to peak was obtained in tissue dialysis. Thus we concluded that the transport from blood into interstitial fluid is very rapid. The mean AUC from both intravenous and subcutaneous microdialysis samples were significantly lower than that from serum samples. We obtained a ratio between intravenous and serum concentrations of 0.50 ± 0.13 suggesting a protein binding of L-dopa of about 50 %. This conclusion is supported by observations by others (85). Pharmacological theories support the view that plasma elimination $T_{½}$ of a compound is independent of protein binding when protein binding is low or moderate. The difference in $T_{½}$ between intravenous microdialysis and ordinary plasma was therefore unexpected. It was more marked in some cases than in others (paper V, Fig 3). I suggest that in some cases there is a strong binding to a protein at low concentrations, which gives rise to a delayed elimination from plasma. There was no difference in $T_{½}$ between microdialysis in blood and subcutaneous tissue.

We compared the results from a group of patients with Parkinson's disease with that of a control group of healthy volunteers. There was a tendency to delay of the mean time to peak value in the patient group. Although the difference was not statistically significant, this raises the

question whether there could be a beginning malfunction of gastric emptying in the patient group (61).

In paper VI we studied the effects of L-dopa treatment on 5-S-cysteinyldopa and on melanoma activation in one patient for five years. L-Dopa increased the concentrations of 5-S-cysteinyldopa in both blood and urine to slightly above the upper normal reference limit (paper VI, Fig. 1). No activation of the melanoma was seen. On two occasions high 5-S-cysteinyldopa peaks were seen but no explanation was found. We conclude that L-dopa given in a single dose gives a significant increase of 5-S-cysteinyldopa within 30 min (paper VI, Fig. 3) but the treatment with L-dopa should not be contraindicated in parkinsonian patients with earlier malignant melanoma.

The patients studied in paper VI had had their parkinsonism for a longer period than those studied in paper V. It is therefore of interest to compare their results. Sorting out the patient with a marked delay in L-dopa absorption I have compiled the pharmacokinetic data of the others in Table 1. The AUC figures from the dialysates seem somewhat higher compared with those from paper V. The ratio between intravenous dialysates and serum was 0.65 supporting the results and conclusions from paper V. We found no differences in $T_{½}$ between the dialysates and serum but it is difficult to draw any statistical conclusions from this small material. Differences in T_{peak} between subcutaneous dialysates and serum were significant.

One patient was studied separately since she had a different pattern of L-dopa pharmacokinetics (paper VI, Fig. 2). The L-dopa peak was much delayed and the concentrations obtained with intravenous microdialysis were very low as compared with both serum samples and subcutaneous dialysates. We suspected delayed gastric emptying time and restudied the patient with concomitant gastric emptying investigation. The late peak and low concentrations of L-dopa were confirmed (paper VI, Fig. 4) and gastric half-emptying time, 191 min, was also delayed (reference interval 47-99 min).

The lower AUC in dialysis compared with those from serum samples (paper V, Table 3; Table 1 below) could indicate a low recovery of L-dopa across the microdialysis membrane. However, *in vitro* recovery determinations at 20 °C were performed after each experiment. The recovery for the intravenous probe was 84 ± 14 % and for the subcutaneous probe it was 82 ± 14 %. We also compared *in vitro* recoveries at 20 and 37 °C using both intravenous (n=4) and subcutaneous (n=4) probes. The recoveries for the intravenous probe were 76 ± 18 (20 °C) and 91 ± 26 (37 °C) and for the subcutaneous probe the recoveries were 75 ± 10 and 75 ±

19 respectively. The results from the intravenous probes agreed well with those obtained with retrodialysis (94 ± 2.9 % and 86 ± 1.8 % in two cases, see paper V). Thus low recovery of L-dopa should not be the explanation of the differences in AUC between plasma samples and dialysates.

Table 1 Pharmacokinetic parameters of L-dopa obtained from four patients with Parkinson's disease and a history of malignant melanoma.

	AUC µmol x min/L	**$T_{½}$ min**	**T_{peak} min**
Intravenous microdialysis	380 ± 121	41 ± 16	98 ± 12
Subcutaneous microdialysis	410 ± 84	54 ± 18	124 ± 38*
Serum	584 ± 119	57 ± 16	75 ± 15

What does microdialysis results reflect?

As shown in paper I (Fig. 5) there is a close contact between the dialysis membrane and the interstitium and melanoma cells of the melanoma. According to physics the low-molecular compounds of the interstitium diffuses through the dialysis membrane into the dialysis fluid. Since the compounds in interstitial fluid are in regulated interchange with the intracellular milieu we believe that the concentrations of the substances collected also may reflect the intracellular concentrations. Thus in paper I we found 5-S-cysteinyldopa concentrations in melanoma microdialysates to be in the range 0.1-1.0 µmol/L while in plasma the concentration usually is in the 1-10 nmolar range. These tumor interstitial concentrations thus reflect the high intracellular 5-S-cysteinyldopa production and concentration of the tumor, and in paper IV a significant correlation was obtained between melanoma tissue homogenates and dialysate concentrations. A better understanding of gradients would be obtained if free plasma and interstitial concentrations could be measured simultaneously in the same animal bearing melanoma.

During the work with paper III and IV we observed a rapid increase of cysteine in the dialysates from melanoma transplants 30 min after a single injection of NAC. Simultaneously we analyzed increasing interstitial concentrations of NAC in the tumor transplants preceding the increase of cys

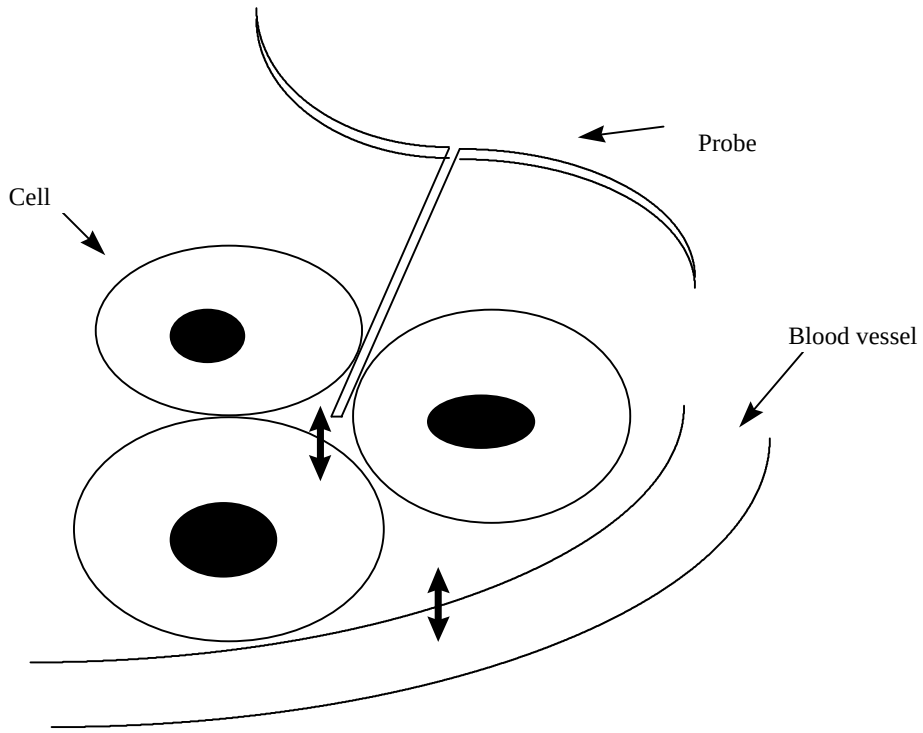

Fig. 4 Microdialysis in extracellular fluid, which is in regulated interchange with both the intracellular milieu and blood circulation. Please note that the sizes of the components (probe, cells and blood vessels) are out of proportion in relation to each other for pedagogic reason.

teine. These findings raised the question whether the extracellular fluid is primarily in balance with the intracellular milieu or with the blood circulation. The rapid appearance of NAC in the tumor interstitium indicates that the extracellular fluid is in direct and rapid interchange with blood circulation.

In paper IV we compared the intracellular concentrations of glutathione, cysteine and 5-S-cysteinyldopa in melanoma tissue with those of tissue dialysates. We found no correlation regarding glutathione or cysteine, but with 5-S cysteinyldopa there was a significant correlation between intracellular levels and dialysate levels from both initial phases and plateau fractions. This may indicate that the interstitial concentrations of cysteine and glutathione are more dependent on the concentrations in the general circulation than in the melanoma cells, and the inverse would be true for 5-S-cysteinyldopa.

In paper V we studied the relation between the subcutaneous extracellular fluid and blood circulation concentrations of L-dopa after oral ingestion. There was a good agreement between the intravenous and the subcutaneous results. Thus, in this case microdialysates from tissue seem to mainly reflect the interchange with blood.

Conclusions

- The microdialysis technique can be applied to the study of metabolites of human melanoma xenografts transplanted to athymic mice. The choice of matrix of the microdialysis membrane is important for proper results. Membranes consisting of cuprophane or polyamide are suitable for studies of interstitial 5-S-cysteinyldopa and selected thiols. Analytical procedures have been improved for quantitation of 5-S-cyteinyldopa, L-dopa, glutathione, cysteine, and NAC.

- For *in vivo* manipulation of glutathione synthesis the human melanoma xenograft model is suitable. BSO greatly suppresses the glutathione concentration in melanoma cells and microdialysates of melanoma tissue. Restitution of glutathione occurs spontaneously and is not improved much by treatment with NAC or OTC. 5-S-Cysteinyldopa in melanoma interstitium is not substantially affected by great variations in glutathione concentrations.

- In the melanoma xenografts the interstitial concentration of 5-S-cysteinyldopa reflects the high intracellular production of this intermediate metabolite. Transport of NAC from intraperitoneal injection to melanoma tissue occurs rapidly and deacetylation of NAC to cysteine *in vivo* can be detected soon after NAC injection. Formation *in vivo* of cysteine is slower from OTC than from NAC.

- The microdialysis technique can safely be applied to intravenous studies of drugs and metabolites in human subjects. With L-dopa the free L-dopa concentration in blood circulation is in rapid interchange with the interstitial subcutaneous concentration. Comparison of microdialysis results with ordinary plasma total concentrations indicates a slight to moderate protein binding of L-dopa. Plasma free L-dopa has similar elimination $T_{½}$ as interstitial L-dopa; in some cases the elimination of total L-dopa is less rapid.

- Difficulties in intestinal resorption of L-dopa after oral intake can be shown by microdialysis in the circulation and subcutaneous tissue. Detailed studies in one case indicate that this may be due to delayed emptying of the stomach.

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