ceed. We envision that variant alleles for the autosomal or X-linked locus (or loci) also exist between *M. poschiavinus* and C57BL/6J, and it is the mismatching of these genes with the Y-linked locus that causes the disruption of the initial testis determination steps.

Further investigation is under way to determine whether the *M. poschiavinus* Y-linked testis-determining gene functions abnormally when transferred to other inbred strain backgrounds (for example, AKR/J, DBA/2J, and BALB/cBy), because the possibility exists that only C57BL/6J carries X-linked or autosomal genes that interact abnormally with the Tdy^{POS} gene. In addition, experiments are in progress to determine whether the *M. poschiavinus* Tdy^{POS} gene functions normally when transferred to other *M. domesticus*, as well as *M. musculus*, genomes. These results will be of special interest because mutations that disrupt the testis determination pathway could be an effective mechanism for initiating speciation.

Mice from the C57BL/6J-Y^{POS} strain will be extremely useful for testing hypotheses concerning sex determination. For example, Wachtel *et al.* (*13*) suggested that the transplantation antigen molecule called H-Y initiates testis development in the uncommitted gonad. In conflict with this hypothesis is our finding that C57BL/6J-XY^{POS} individuals with two ovaries are positive for H-Y as determined by skin grafting, cell-mediated lympholysis, and the popliteal lymph node assays (*14*). Finally, the availability on a common genetic background (C57BL/6J) of two Y chromosomes that differ at their testis-determining locus will be instrumental in identifying Y-linked DNA sequences involved in primary sex determination.

Note added in proof: We have found that three Y chromosomes derived from *M. domesticus* cause sex reversal after transfer to the C57BL/6J genome. The three Y chromosomes were from mice trapped in northern Italy (Alpi Orobie, near Bergamo), central Italy (Apennine, Molise), and southern Italy (Lipari, Isole Eolie). In all cases, XY hermaphrodites were recovered in the first backcross generation. Thus, the Y-linked sex reversal phenomenon observed with the Y^{POS} chromosome is the result of transferring the *M. domesticus* Y chromosome to the C57BL/6J genome.

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Intracellular Oxidation-Reduction State Measured in situ by a Multichannel Fiber-Optic Surface Fluorometer

Abstract. *The principles of the measurement in vivo of the oxidation-reduction state of intramitochrondrial pyridine nucleotides were used in establishing a multichannel fluorometer-reflectometer. This approach made possible the study of changes of mitochrondrial redox states in four different organs (brain, liver, kidney, and testis) of the same animal, as well as the monitoring of four different cortical areas of the same brain hemisphere. In the measurement of reduced nicotinamide adenine dinucleotide fluorescence, oximetric and movement artifacts are negligible, but blood volume changes and tissue absorption properties are a source of error. The corrected fluorescence is obtained by subtracting the reflectance from the fluorescence signal in 1:1 ratio. During graded hypoxia, the corrected fluorescence showed a gradual increase and was maximal during anoxia in all four organs tested.*

The first detailed study on surface microfluorometry of organs in situ was reported in 1962 by Chance *et al.* (*1*). Since then, the same basic approach has been used to study the oxidation-reduction states of various tissues in various animal models, including the human brain [for review, see (*2*)]. Because there have been discrepancies in results, as well as in their interpretation (*3*, *4*), we discuss in detail results obtained with fiber-optic fluorometry-reflectometry during the last 9 years.

Since the first light-guide fluorometer was built at the end of 1972, fiber optics have been used in various types of fluorometers (*5*, *6*). The direct-current (d-c) fluorometer-reflectometer containing a Y-shaped light guide has been of value in most of the studies in which reduced nicotinamide adenine dinucleotide (NADH) fluorescence is measured. Mayevsky and Bar-Sagie (*7*) described the use of the two-channel d-c fluorometer-reflectometer with dual Y-shaped light guides in the study of brain energy metabolism. We now describe the use of the four-channel d-c fluorometer-reflectometer to monitor four different organs and also to monitor four different locations on the same organ.

The principles of the single-channel d-c fluorometry are shown schematically in Fig. 1. In the present four-channel fluorometer, this unit was quadrupled, and small variations were made in the light guide, as described below. The light source was a 100-W water- or air-cooled mercury arc having a 366-nm filter in front of it between the fluorometer and the excitation bundle of the fibers. The light guide contains four bundles of excitation fibers split from the light source; another four bundles of fibers transmitting the emitted light form four Y-shaped light guides. We used quartz fibers having a diameter of 2 mm in each common part, as well as plastic ones having a diameter of 0.8 mm in each common part. The emitted light from the tissue

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was split in a ratio of 90:10 and was used to measure fluorescence at 450 nm and reflectance at 366 nm, respectively. We corrected for artifacts in the NADH fluorescence measurement by using a 1:1 subtraction technique to obtain the corrected fluorescence trace. The four-channel fluorometer was used to obtain recordings in five rats and five gerbils. In the rat, we monitored NADH in the brain, liver, kidney, and testis simultaneously; in the gerbil we monitored four points on the same brain hemisphere. Animals were anesthetized by intraperitoneal injection of Equithesin (a mixture of pentobarbital, chloral hydrate, magnesium sulfate, and propylene glycol). The light guide monitoring the brain was connected by a holder cemented to the skull with dental acrylic (*6, 7*).

Figure 2, obtained from a rat ventilated with a respirator after intravenous injection of Flaxedil to stop spontaneous breathing, shows the effects of graded hypoxia and anoxia on the reflectance and corrected fluorescence. The uncorrected fluorescence was measured from each organ but was not recorded on the same chart paper. A typical response to hypoxia, namely an increase in the corrected fluorescence and at the same time a decrease of reflectance, was obtained when the rat was ventilated with 10 percent oxygen (in nitrogen). The largest change in the corrected fluorescence signals was measured during complete deprivation of oxygen, achieved by ventilating with 100 percent nitrogen. The response to 5 percent oxygen was intermediate; thus, there appears to be a direct correlation between the partial pressure of oxygen (PO_2) in the air breathed and the magnitude of change in the NADH redox state, as previously described by Chance *et al.* (*1*). (The results were the same when the rat was breathing the same low-oxygen mixtures spontaneously.) These typical responses were found in all four organs tested, with little variation between them. In all of the normal rats tested (more than 1500 animals in nearly 9 years), the same qualitative response to anoxia was found in the normoxic brain.

The magnitude of the decrease in the reflectance signal was not greatly affected by the level of PO_2, probably because autoregulating vasodilation takes place during hypoxia to increase blood volume to the organ. This vasodilation was activated even by ventilating the animal with 10 percent oxygen, and a decrease in the reflectance was recorded. In the testis, the reflectance response was minimal and was probably due to the small amount of vascularity in the measurement site (observed during the exposure of the organ). The kidney showed an atypical reflectance response in this animal; that is, when the rat breathed nitrogen only, a large increase in reflectance was recorded simultaneously with the expected large increases in the corrected fluorescence. The recovery of the reflectance to its base line was very slow (not shown); this response was not observed in all kidneys tested. The same animal was exposed to anoxic cycles a few times before this record was taken, and this large change in reflectance was not found earlier. The kidney of this animal did not recover to its normal state (Fig. 2B).

In the five animals used in this part of the study, the kinetic responses in NADH fluorescence during the transition from the normoxia to anoxia were different in the four organs tested. When the respirator was turned off (Fig. 2B), the reflectance and the corrected fluorescence traces showed the same pattern as was found in the transition from normoxia to anoxia, but with a longer lag period resulting from the oxygen left in blood and tissues. The transition from anoxia to normoxia (by respirating the rat) was clearly faster, except in the kidney, where the microcirculation system had probably been damaged earlier (Fig. 2A). More experiments are required to explain the variation between the different organs.

Figure 3 shows a typical response of the gerbil brain to anoxia and cortical spreading depression. These responses were found in all normoxic gerbil brains (more than 300 gerbils tested during the last 3 years). The animal was breathing air spontaneously, and when it was exposed to 100 percent nitrogen, a typical two-step decrease in reflectance was recorded together with a large increase in corrected fluorescence, which reached a plateau shortly thereafter. When air breathing was restored, a fast reoxidation of NADH was recorded. In this gerbil the anoxia induced a secondary response characterized by an oxidation cycle of NADH appearing first in site 1, then propagated to site 4 through sites 2 and 3. This phenomenon was a spreading depression initiated during the anoxia by the intrinsic elevated extracellular K^+ ions (*8*). The same response to spreading depression was monitored after initiation of a wave by application of KCl (Fig. 3B). When the wave reached site 1 (the one closest to the KCl application site), a typical biphasic change in the reflected light was recorded. The corrected fluorescence shows an oxidation cycle lasting between 1 and 2 minutes, depending on the measuring site in relation to the propagation front of the spreading depression. The reflectance response had a

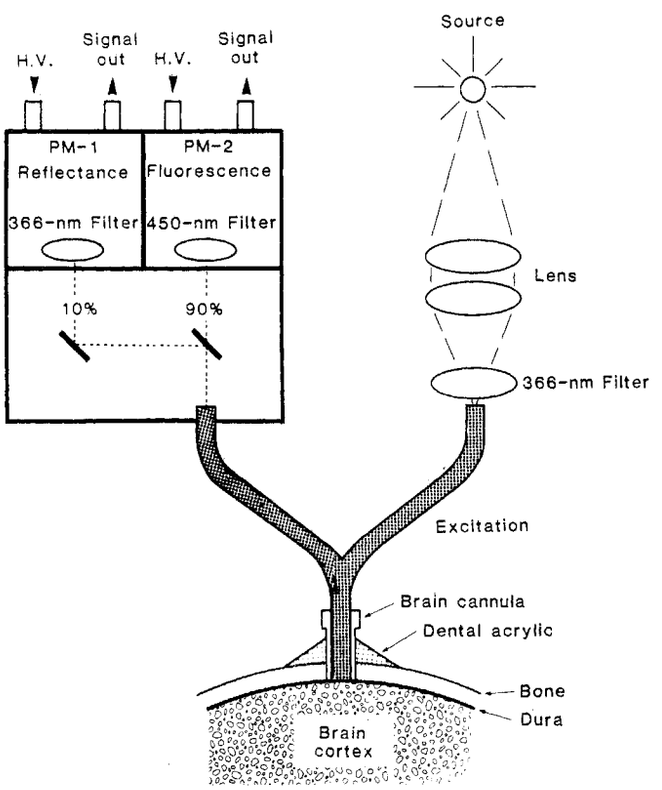

Fig. 1. Schematic presentation of the single-channel d-c fluorometer-reflectometer, which served as the basis for the multichannel instrument used in the present study. *H.V.*, high-voltage input; *PM-1* and *PM-2*, photomultipliers.

short increase phase, followed by a very long decrease below base line and then a recovery phase.

Four sources of error, which may affect the measurement of NADH fluorescence in vivo, are (i) movement artifacts; (ii) changes in the oxygenation level of the blood; (iii) changes in the absorption properties of the tissue monitored; and (iv) blood volume changes in the tissue under observation. We found that when a good contact is made between the bundle of fibers and the brain (or any other organ), all movement artifacts are eliminated or avoided. The oxy-deoxy hemoglobin absorption changes are minimal in our system (*9–12*). Little is known about the changes in the absorption properties of the tissue during the various perturbations of the observed measuring site, since it is difficult to separate these changes from other factors affecting the NADH fluorescence reading. We have shown indirectly that under physiological or pathological conditions where there is movement of ions and water between the intracellular and the extracellular space, changes in the absorption properties of the tissue being monitored will have an effect on NADH fluorescence measurements, and we have corrected for this effect by the subtraction technique (*13–16*).

Various investigators have attempted to correct for changes in the blood volume of the tissue under observation. Aubert *et al.* (*17*), using slices of the electric organ of the electric fish, monitored the reflected light. Jobsis *et al.* (*18*) used the reflected light at 366 nm and found that 1:1 subtraction was suitable for many brain preparations, although not for all. Using a fiber-optic fluorometer, we found in 1973 that 1:1 is a good correction factor for most of the brains tested by fast injection of saline into the common carotid artery (*19*). This approach is today the dominant one for surface fluorometry in vivo (*7, 16, 19–21*). Dora and Kovach (*22*) described the correction factor problem in monitoring cat brain NADH in vivo; they are using the saline flush technique to check and use a variable correction factor. Kramer and Pearlstein (*4*), using an isosbestic microfluorometer rather than the 366-nm reflectance fluorometer, reported that the typical response to anoxia was "unexpected initial early oxidation," although an explanation for this finding was not given. Under anoxia, the kinetics of the corrected fluorescence would be expected to be similar to that of mitochondria, and this has been observed by many investigators. We believe that the correction factor, as well as the exact mathematical equation to be used for the correction, is dependent on the optical system used in the fluorometer and on the relation between the optical system and the tissue being monitored.

Activation of the mitochondria by adenosine diphosphate may result in an oxidation of NADH (*23*), and indeed most investigators have shown that direct cortical stimulation in vivo, convulsions, and spreading depression lead to the oxidation of the brain NADH (*2, 7, 10, 18, 21, 24*).

The response of the brain to spreading depression can be used as an indicator for the intactness of the hemodynamic and metabolic compensation mechanisms in the brain (*25*). The pattern of changes of the reflectance trace is a good indicator of the hemodynamic responses. Hence, the use of a short anoxia cycle (30 to 40 seconds) as well as a

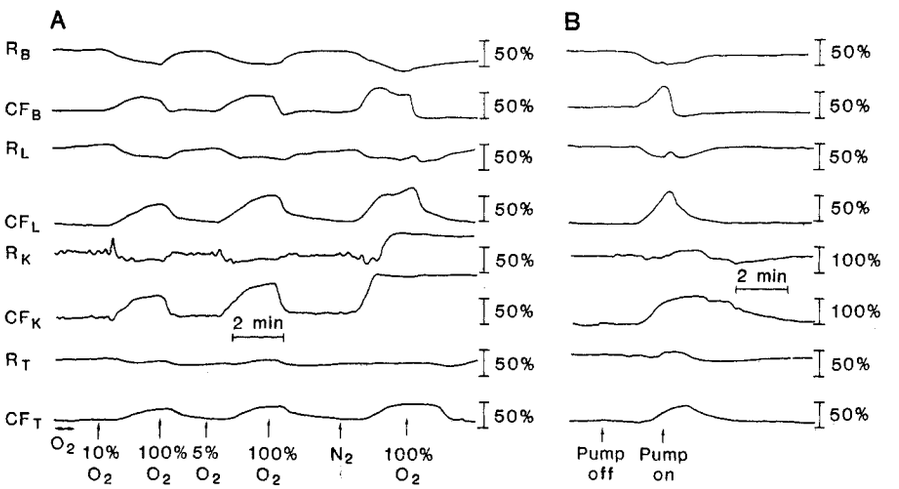

Fig. 2. (A) Effects of graded hypoxia and anoxia on the NADH redox state in an artificially ventilated rat. Four organs were monitored simultaneously, and for each organ we recorded the reflectance (*R*) and the corrected fluorescence (*CF*). Subscripts: *B*, brain; *L*, liver; *K*, kidney; and *T*, testis. (B) Effects of asphyxia.

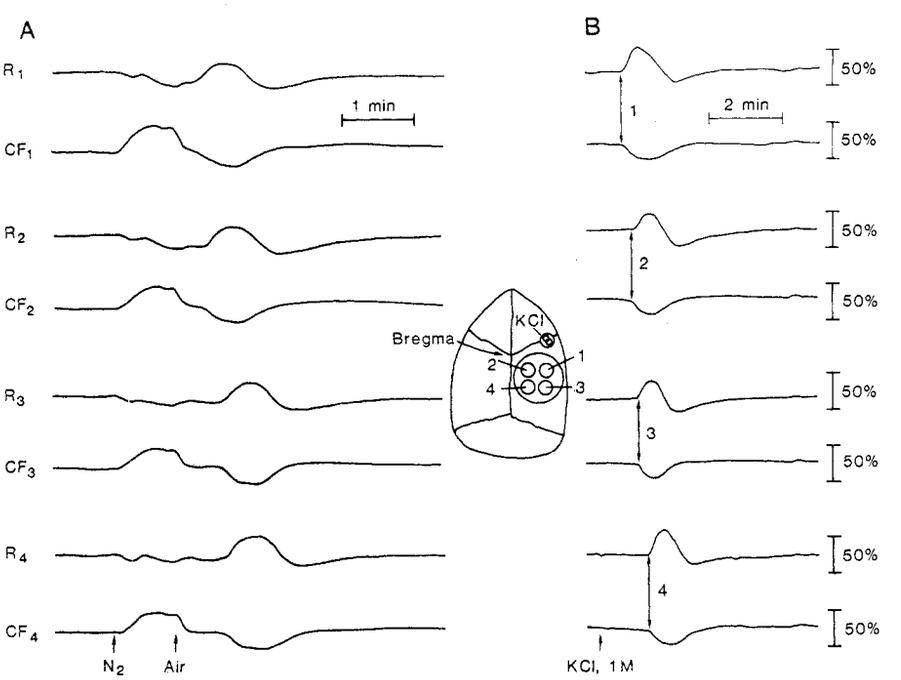

Fig. 3. Metabolic responses of a gerbil brain to (A) anoxia and (B) cortical spreading depression in four different locations in one hemisphere. Arrows 1 to 4 in (B) show the propagation of the spreading depression throughout the measured area. The schematic drawing between (A) and (B) shows the locations of the four light guides above the parietal cortex area.

spreading depression can disclose the functional state of a brain.

The use of the multiple organ recording was started when Chance *et al.* (*1*) monitored NADH fluorescence in brain and kidney simultaneously. This approach was essential in the initial studies of organ anoxia by the fluorescence method. Our present study expands their idea and gives a strong justification to the use of surface fluorometry–reflectometry.

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Tumor Cell–Platelet Aggregation: Induced by Cathepsin B–Like Proteinase and Inhibited by Prostacyclin

Abstract. *The ability of tumor cells to metastasize may be related to their ability to promote aggregation of host platelets. The use of inhibitors of cysteine proteinases resulted in parallel inhibition of B16 amelanotic melanoma–induced platelet aggregation and of cathepsin B activity. The antimetastatic agent prostacyclin inhibited platelet aggregation induced by the tumor cells and by papain, a cathepsin B–mimicking agent.*

Abnormalities in blood coagulation are common in patients with advanced malignant disease (*1*). Tumor cells have platelet aggregating activity (*2*) as well as procoagulant activity (*3*). A tumor cell procoagulant activity responsible for alterations in the fibrin-fibrinogen system was shown to result from a cysteine proteinase (*3*). The ability of tumor cells to enhance aggregation of platelets in vitro correlates positively with their metastatic potential in vivo (*2*), and manipulation of host platelet levels by induced thrombocytopenia decreases metastatic tumor colony formation in some systems (*2*). Prostacyclin (PGI_2), the most potent agent known for preventing platelet aggregation, significantly inhibits metastasis of the murine B16 amelanotic melanoma (B16a) (*4*).

Local proteolysis by proteinases released into the extracellular matrix of tumors has been implicated in tumor invasiveness and metastasis (*5*). A cathepsin B–like cysteine proteinase has been shown to be released from tumors (*6, 7*), and cathepsin B levels are elevated in the serum of patients with several varieties of cancer (*7*). We have found that tumor cell levels of a cathepsin B–like cysteine proteinase correlate positively with the metastatic potential of the B16 melanotic melanoma (*8*) and that both the B16a and the B16 melanotic melanoma in culture release cathepsin B into the media (*8, 9*). We now describe studies designed to determine whether a tumor cell–derived cathepsin B–like cysteine proteinase is responsible for B16a tumor cell–induced platelet aggregation and whether this effect can be inhibited by PGI_2.

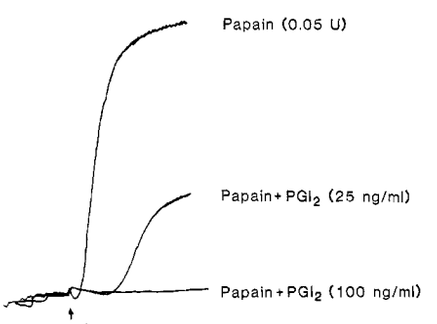

Fig. 1. Inhibition of papain-induced aggregation of washed human platelets by prostacyclin. Aggregometer tracings of human platelet aggregation. The upward deflection of the pen indicates an increase in light transmission due to platelet aggregation. The time scale from the arrow to the end of the tracing is 5 minutes. Washed platelets were prepared from human platelet-rich plasma by the method of Hamberg *et al.* (*21*). Platelets were counted (Coulter counter, model ZBI), and the final count was adjusted to $5.0 \pm 0.5 \times 10^8$ platelets per milliliter. To the aggregometer cuvette were added 250 μl of the washed platelet suspension, 5 μl of 0.1*M* $CaCl_2$, and 5 μl of PGI_2 or its diluent (0.025*M* glycine and 0.025*M* NaCl, *p*H 10.5). One minute after addition of PGI_2 or diluent, 5 μl of papain was added, as indicated by the arrow. The aggregation was determined at 37°C with a dual-channel aggregometer (Sienco) at a stirring speed of 800 rev/min. Prostacyclin (100 ng/ml) completely inhibited papain-induced aggregation.

Subcutaneous B16a tumors were maintained in syngeneic C57BL/6J mice (Jackson Laboratory). Tumors were disaggregated, and the tumor cells were purified by centrifugal elutriation (*8*). Elutriated tumor cells (>90 percent viability) were used for platelet aggregation studies or were homogenized and assayed fluorometrically for cathepsin B activity and protein (*8*). Cathepsin H was assayed with L-Arg-4-methoxy-β-naphthylamine (L-Arg-4-OMe-β-NA) (Arg, arginine) or L-Arg-aminofluorocoumarin (L-Arg-AFC; Enzyme Systems Products, Livermore, California) as substrate, at *p*H 6.8 (*10*). Enzyme assays were standardized to ensure linearity.

Proteinase inhibitors of varying specificity for both cysteine and serine proteinases (*11*) were tested for their ability to inhibit B16a-induced aggregation of washed human platelets. The most effective inhibitors of B16a-induced aggregation of washed human platelets were leupeptin and antipain (Sigma) (Table 1). Umezawa (*12*) showed that leupeptin inhibits the cysteine proteinase activity of cathepsin B and papain at a median inhibitory dose (ID_{50}) of 1 μ*M*; the ID_{50} for inhibition of thrombin activity is 20 m*M*. Iodoacetic acid (IAA; Sigma) and *p*-hydroxymercuriphenylsulfonate (PHMS; Sigma), specific inhibitors of cysteine proteinases, both inhibited B16a-induced platelet aggregation. *N*-

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