virally and chemically transformed HOS cells. Fluorescein-conjugated rabbit antiserum to rat immunoglobulin G (Microbiological Associates) was used at a dilution of 1:15. Indirect immunofluorescence in cultures of both types of transformed cells, treated with cycloheximide or not, was scarcely visible with their antiserums. Immunofluorescent staining of proteins on the surfaces of both types of transformed cells could be observed. However, the cell surface antigens could not be detected in virally or chemically transformed HOS cells treated with cycloheximide. The immunofluorescence index (*15*) was less than 30 percent (considered negative) in both types of treated cells.

Our results demonstrate that the addition of cycloheximide (0.08 μg/ml) in culture medium caused both virally and chemically transformed human osteosarcoma cells (TE-85, clone F-5) to revert temporarily to normal phenotype, but the same amount of cycloheximide did not affect the rate of growth of untransformed cells. Increasing the amount of inhibitor from 0.08 to 1 μg/ml strongly inhibited normal cell growth, suggesting that the primary effect of this protein inhibitor on both types of transformed cells may be one of nonspecific toxicity. The mechanism by which cycloheximide maintains the flat morphology in the transformed cells is not understood. Regulation of the expression of either oncogenic RNA virus or chemical carcinogen transforming gene sequences might be under the influence of a translational control mechanism because of a translational inhibitor-dependent reversion in both types of transformed cells. Demonstration of surface antigens, cross-reacted with antiserums induced by extracts of both types of transformed cells, also depended on the presence or absence of cycloheximide in the culture medium. These cell surface antigens (*16, 17*) are clearly associated with the process of transformation, and whatever mechanism was involved in the transformation, the malignant state could be reversed by an inhibitor of protein synthesis. These results show that protein synthesis is required to maintain the transformed state in chemically and virally transformed human cells. Synthetic inhibitors of proteases have been used successfully to suppress 7,12-dimethylbenz[*a*]anthracene-induced and phorbol ester-promoted tumorigenesis in mouse skin (*18*). The possibility of accomplishing the same thing in vivo would have exciting implications for the management of human cancers.

Cycloheximide has also been shown to induce type C virus at high frequency from virus-negative mouse cells by an increase in the cellular concentration of type C viral RNA in cycloheximide-treated cells (*19*). However, no type C virus induction was observed in the cycloheximide-treated chemically altered human cells.

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Cerebral Cortical Microfluorometry at Isosbestic Wavelengths for Correction of Vascular Artifact

Abstract. *Microfluorometric measurements of cerebral cortical mitochondrial respiration in vivo are obscured by hemodynamic and oximetric artifacts. Isosbestic fluorometry provides appropriate correction for these vascular phenomena and permits simultaneous evaluation of mitochondrial nicotinamide adenine dinucleotide redox state, microcirculatory volume, and hemoglobin oxygenation.*

Tissue microfluorometry was originally introduced as an optical method whereby mitochondrial respiration could be examined continuously and nondestructively in vivo (*1*). The transducible property underlying the technique is the intense blue fluorescence (440 to 470 nm) produced when reduced mitochondrial nicotinamide adenine dinucleotide (NADH) is excited by light in the near-ultraviolet portion of the spectrum (340 to 370 nm); the oxidized cofactor (NAD), as well as unbound or cytoplasmic NADH, exhibits negligible fluorescence (*1, 2*). This property was initially exploited in studies of the NADH : NAD redox state in isolated mitochondria by Chance *et al.* in the early 1950's, and soon thereafter microfluorometry was applied to metabolic studies of intact tissue in situ (*1*). Subsequently, numerous investigators have variously modified the method to examine mitochondrial behavior in heart, liver, kidney, thyroid, and brain (*3*).

Cerebral microfluorometry, developed most fully by Jobsis and co-workers (*4*), has been used to provide a qualitative indication of the kinetics and efficiency of cortical mitochondrial respiration during a variety of experimental interventions in both laboratory animals and in humans (*5*). On review, it is apparent that tissue microfluorometry is no longer a single instrumentation system as initially described by Chance *et al.* but a family of distinct systems, each based on the same transducible property (NADH fluorescence), yet each reflecting a distinct opinion concerning significant technical problems that compromise fluorometric analysis in situ. These problems appear to be inherent in tissue microfluorometry and have produced increasing uncertainty over both the proper application of the method and its utility in general (*6*).

Persuaded that the potential of the fluorometric method is not yet fully realized, we have studied in considerable detail the optical properties of cerebral cortical tissue in order to identify and eliminate the artifacts that most seriously compromise the use of fluorometry in

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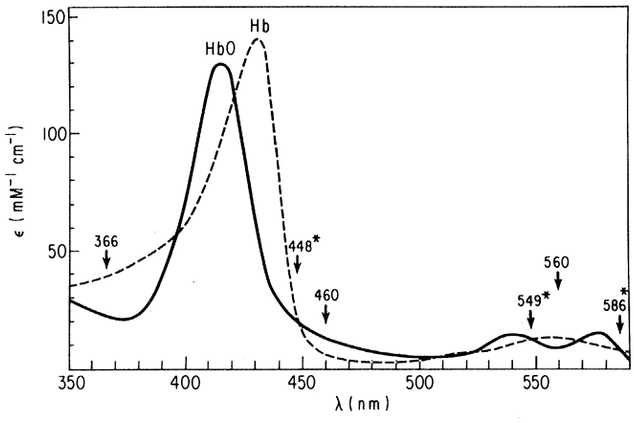

Fig. 1. Absorption spectra of oxygenated (*HbO*) and deoxygenated (*Hb*) hemoglobin. Wavelengths (λ) referenced in the text are specifically indicated; asterisk denotes relevant isosbestic wavelengths—that is, those at which the extinction coefficients (ε) of the two species are equal.

such studies. In the course of these investigations, we have evolved an instrumentation system, which we refer to as isosbestic fluorometry. This technique appears to possess distinct advantages over other systems suggested to date. First, it provides an accurate (and, we believe, quantitative) measure of NADH fluorescence in the cerebral cortex both at rest and during metabolic perturbations. Second, the method simultaneously indicates hemoglobin oxygenation state in the monitored field. Finally, the system is calibrated with tissue fluorescence parameters; external references are not necessary for operation.

Three interdependent physiological phenomena obscure the measurement of a fourth, NADH fluorescence, during studies of anoxia or other metabolic interventions in situ. These may be denoted the movement artifact, the hemodynamic (blood volume) artifact, and the oximetric artifact.

Movement artifact is observed most prominently in large animals as a consequence of the increased volume and compliance of the subarachnoid space, which substantially enhance respiratory and arterial pulsatile movements observed in the exposed, healthy brain and produce significant changes in the geometric relationship between the cortex and the optical system.

Hemodynamic artifact occasioned by autoregulation is more difficult to control than movement. Because blood strongly absorbs both the excitation wavelength (366 nm) and the fluorescence signal of interest (~ 460 nm), changes in cerebral blood volume of the sort that invariably accompany any metabolic intervention profoundly affect tissue fluorescence measurements.

Finally, one must contend with the artifact created by the oximetric effect of hemoglobin, resulting from its changing absorption spectrum at various oxygenation levels during an evolving anoxic insult or other metabolic stress; we now recognize the oximetric transition as a major determinant of signal intensity at nearly all wavelengths.

Most investigators have attempted to resolve these problems by simultaneously monitoring an indifferent wavelength remote from the emission spectrum of NADH. In nearly all cases they have elected to measure the intensity of the 366-nm excitation light reflected from the cortical surface, then (after electronically setting the two output signals to unity in the baseline or normoxic state) to algebraically subtract reflectance from the NADH fluorescence signal (*3*-*5*). In our experience the reflected 366-nm signal provides inadequate compensation for movement and hemodynamic artifact and little or no correction of oximetric artifact. We have observed that a substantial component of the 366-nm signal is provided by specular (and to a lesser extent diffuse) reflectance from the membranous arachnoid, the single biological element in the system most susceptible to movement secondary to the ebb and flow of spinal fluid, and anatomically interposed between the optics and the vascular channels for which one is attempting to provide correction. If meticulous attention is paid to positioning of the optics, movement artifact may be minimized but hemodynamic and oximetric artifacts persist. Kovach and co-workers (*6*) recently described in detail the difficulties attendant on use of the 366-nm reflectance as a reference signal.

Furthermore, it appears to us that subtraction of the reference signal from the signal of interest is inappropriate; the relationship between the two normalized signals is properly a ratio. This is best illustrated by hypothesizing an extreme case in which NADH reduction and autoregulatory vasodilatation could be made to occur independently; if a massive increase in blood volume were sufficient to obscure both signals 90 percent

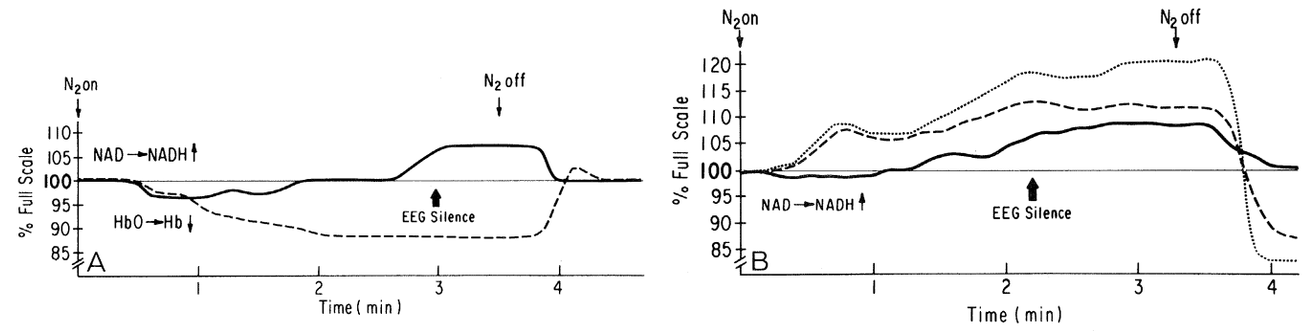

Fig. 2. (A) Isosbestic fluorometry applied to the measurement of mitochondrial NADH fluorescence (448/549 nm, solid line) in canine cerebral cortex during experimental anoxia. The HbO → Hb oximetric transition (560/549 nm, dashed line) within the microvasculature is recorded simultaneously. Solid arrow indicates the onset of cortical isoelectricity (animal anesthetized with pentobarbital). Oximetric insensitivity of the 448/549-nm signal ratio is reflected in the independent kinetics of the two phenomena, with substantial NAD reduction appearing 30 seconds after hemoglobin desaturation is complete. Abbreviation: *EEG*, electroencephalogram. (B) Comparison of traces obtained simultaneously during canine cerebral hypoxia, using isosbestic (448/549 nm, solid line), traditional (448 minus 366 nm, dashed line), and modified traditional (448/366, dotted line) fluorometry. The initial oxidation of NADH detected at 448/549 nm is not observed when 366-nm reflectance is employed as the reference wavelength. Possible oximetric distortion of the 366-nm signal is suggested by similarities between the 448/366-nm kinetics in this illustration and the reciprocal of the 560/549-nm (deliberately oximetric) trace in (A).

followed by doubling of the NADH fluorescence, algebraic subtraction would mistakenly interpret the difference between the reference and fluorescence signals (10 and 20 units, respectively) as 10 units or 10 percent of initial NADH reduction. Application of the ratio technique would give the correct interpretation.

Several investigators have recognized the oximetric transition of hemoglobin as a potential problem in fluorometry, albeit without attempting to quantitate the effect. It should be recalled that the absorption spectra of oxy- and deoxyhemoglobin are quite distinct, although if one is superimposed on the other, wavelengths are found at which the extinction coefficient is identical for the two molecules. These are referred to as isosbestic wavelengths; all other wavelengths are therefore more or less anisosbestic (Fig. 1).

Chance *et al.* (*7*) observed that the 366-nm reflectance signal was significantly anisosbestic, but felt that the deviation from isosbesticity was proportional to that of the signal of interest; thus the two signals could be considered isotransitional. However, this rationalization is at least theoretically inadequate, largely as a consequence of the nonlinear spectral absorption characteristics of suspended particulate absorbers such as red cells (*8*). Harbig *et al.* (*9*) recorded the signal of interest (NADH) at an isosbestic wavelength, but by retaining the anisosbestic 366-nm reflectance as reference the artifact was only slightly diminished. They also introduced an imaginative approach to blood volume correction by bolus perfusion (hemodilution) of the optical field with calibration of the two signals to unity at the peak of signal enhancement; however, it can be shown that the 1:1 ratio so imposed on the respective signals no longer prevails with changes in blood volume or deviations from the metabolic steady state. Kobayashi *et al.* (*10*) attempted to correct for both oximetric artifact and red blood cell light scatter by applying an independently derived nonlinear correction to the signal of interest, a solution compromised by the use of reflectance and by the nonquantitative nature of the fluorometric record itself.

Early studies in our laboratory in which the 366-nm reflectance signal was employed as a reference yielded unacceptably inconsistent results. On the other hand, differential excitation spectra of the cortex led us to conclude that the cortical fluorochrome population was indeed heterogeneous and that one or more fluorescent species might be metabolically inert. We therefore reasoned that the optimal reference wavelength for correction of hemodynamic and movement artifacts (as well as variable tissue absorption and light scatter) might be found within the fluorescence spectrum of brain itself, if indeed any portion of that spectrum could be identified which was minimally contaminated by NADH fluorescence and was otherwise unresponsive to metabolic influences. Detection of an appropriate wavelength was, of course, complicated by the fact that movement, blood volume, hemoglobin oximetry, and NADH:NAD redox state are more or less mutually dependent.

For this reason we began by creating metabolically inert fluorescence signals in brain tissue through the introduction of artificial fluorochromes; ultimately, rhodamine B was found to possess the properties of an ideal reference fluorochrome. Administered systemically, rhodamine (like NADH) is a pure tissue marker, diffusing rapidly and homogeneously into brain tissue. Its presence in large blood vessels cannot be detected fluorometrically. Fluorescence efficiency is *p*H-independent in the physiological range; it is nontoxic in the doses employed and exerts no apparent metabolic effect on the brain; and it is cleared from tissue very slowly (approximately 1 to 2 percent per hour). Most important, its emission spectrum, when excited by 366-nm light, is far removed from that of NADH; rhodamine fluorescence maximum = 589 nm. In fact, very little intrinsic fluorescence is detectable in brain in this wavelength region.

We therefore assumed that changes in rhodamine-enhanced brain fluorescence at 589 nm would accurately reflect blood volume changes (and movement) but would be unresponsive to metabolic influences. By using different narrow-band interference filters, each region of the intrinsic fluorescence spectrum of brain was examined during simultaneous measurement of the 589-nm (rhodamine) peak. The normalized signals at all wavelengths tested remained equal during both induced vasodilatation (5 percent CO_2 in air) and vasoconstriction (drug-induced, using agents without any known effect on cerebral metabolism).

However, when wavelengths in the region 530 to 550 nm were compared with the 589-nm rhodamine signal during an anoxic intervention (N_2 breathing), substantial divergence of the normalized signals was observed; the character of the deviations could not be correlated with reduction of NAD, but could be related directly to the capillary hemoglobin (Hb) absorption spectrum in the HbO → Hb transition; that is, an oximetric artifact. We consequently chose to examine a known isosbestic wavelength in the region 530 to 550 nm (specifically, 549 nm) together with an isosbestic wavelength (586 nm) within the rhodamine spectrum. The filters selected were tested spectrophotometrically with solutions of oxy- and deoxyhemoglobin and were confirmed to be isosbestic. Recording at these two wavelengths, transitions from normoxia to total hypoxia associated with massive vasodilatation resulted in perfect congruence of the two normalized signals; that is, the 549-nm intrinsic fluorescence of cortex appears to be both metabolically inert and oximetrically insensitive, a pure blood volume and movement indicator. The congruence of the observed signals under these circumstances also indicates that changes in light scattering or in the absorption properties of the various cytochromes during the anoxic transition are not fluorometrically detectable.

Finally, an isosbestic wavelength was selected within the known NADH spectrum (448 nm) and the signal referenced by ratio to the 549-nm signal, with the result that neither the NADH nor the reference signal is contaminated by oximetric influences. Furthermore, when we simultaneously measured a deliberately anisosbestic wavelength within the non-NADH fluorescence spectrum of the tissue (560 nm) and referenced the output to the 549-nm signal, we were able to qualitatively record subtle oximetric transitions in the capillary blood.

The instrumentation used is a modification of the time-sharing fluorometer described by Chance *et al.* (*7*) except that the 366 (± 5) nm excitation waveband from a 100-W regulated mercury arc lamp is separately directed on a small (< 5-mm diameter), relatively avascular area of the cortical surface. The cortical fluorescence spectrum is conducted through a nonfluorescent quartz fiber-optic cable to a time-sharing fluorometer, which permits simultaneous monitoring of up to four different wavelengths by a single high-sensitivity photomultiplier tube. The output of the photomultiplier is gated, discriminated, and electronically processed both to magnetic tape and to a multichannel polygraph recorder. The calibrated taped analog signals are later subjected to analog-to-digital conversion and the output ratios 448/549 nm and 560/549 nm are derived point-for-

point by laboratory computer and plotted.

A typical result obtained when isosbestic fluorometry is employed in the study of cerebral cortical anoxia in dogs is shown in Fig. 2A, which simultaneously depicts the NADH redox state and capillary hemoglobin oxygenation state (the blood volume trace is omitted for clarity). The behavior of NADH during the anoxic transition is multiphasic and includes an unexpected initial early oxidation. The details of the experimental model and our interpretation of the behavior of the mitochondrial respiratory chain during anoxia will be described elsewhere (*11*).

For comparison, Fig. 2B illustrates the simultaneous measurement of 448 minus 366 nm (traditional method), 448/366 nm (traditional method, but imposing ratio analysis), and 448/549 nm (isosbestic fluorometry). During the early stages of the hypoxic response, the 448/366-nm trace resembles a reciprocal of the 560/549-nm (HbO → Hb) transition depicted in Fig. 2A; it may be inferred that the traditional method is compromised both by inadequate blood volume compensation and by high sensitivity to oximetric artifact, resulting in an apparent monotonic reduction of NAD far in excess of that observed by isosbestic fluorometry.

We also observed maximal oxidation of mitochondrial NADH in canine cortex during insulin-induced profound hypoglycemia (plasma glucose < 20 mg per 100 ml; cerebrospinal fluid glucose < 10 mg per 100 ml). These experiments simulate Chance and Williams's (*12*) mitochondrial state 2—that is, substrate depletion in vivo—and together with studies using uncouplers and selected respiratory inhibitors, indicate that in the basal state (before anoxia) mitochondrial NADH is ~ 66 percent reduced and that ~ 77 percent of the basal fluorescence signal at 448 nm is metabolically inert (non-NADH) contaminant fluorescence (*11*).

Our experience suggests that tissue fluorometry provides unique information for the study of mitochondrial behavior in situ and, when employed in the technical configuration that we have, called isosbestic fluorometry, may ultimately permit quantitative interpretation of the fluorescence signal.

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4-Aminobutyrate:2-Oxoglutarate Aminotransferase in Blood Platelets

Abstract. *Platelet lysates obtained from blood of humans, dogs, and rats catalyzed the transamination of 4-aminobutyrate with 2-oxoglutarate as cosubstrate. Human platelet 4-aminobutyrate:2-oxoglutarate aminotransferase (36.5 ± 3.2 picomoles per minute per milligram of platelet protein) resembled the brain enzyme in kinetic properties and in response to cofactors and inhibitors.*

The enzyme 4-aminobutyrate:2-oxoglutarate aminotransferase (E.C. 2.6.1.19) is principally responsible for catabolism of the putative inhibitory neurotransmitter γ-aminobutyric acid (GABA) in mammalian brain (*1*). This enzyme catalyzes the transfer of an amino group from GABA to 2-oxoglutarate, yielding glutamic acid and succinate semialdehyde. The latter may then be oxidized to succinic acid by mitochondrial succinate-semialdehyde dehydrogenase. Although GABA and GABA aminotransferase were formerly thought to be localized in central nervous system (CNS) tissue, more recent work has indicated that GABA metabolism does occur in some peripheral organs, including liver and kidney (*2, 3*). We report the presence of substantial amounts of the catabolic enzyme GABA aminotransferase in blood platelets and compare the properties of the human platelet enzyme with those of the human brain enzyme. If the enzyme activity of platelet GABA aminotransferase can be assumed to reflect brain enzyme activity, assays of the easily obtainable platelet enzyme may

Table 1. Comparison of human brain and platelet GABA aminotransferase.

Property	Human brain*	Human platelets
K_m (GABA)†	$3.1 \times 10^{-4} M$	$3.1 \times 10^{-4} M$
K_m (2-oxoglutarate)	$1.6 \times 10^{-4} M$	$0.70 \times 10^{-4} M$
Effect of pyridoxal phosphate	Stimulates	Stimulates
Inhibition (in vitro) by‡		
Aminooxyacetic acid ($10^{-8} M$)	69 percent	79 percent
Aminooxyacetic acid ($10^{-6} M$)	100 percent	97 percent
Ethanolamine *O*-sulfate (0.3 m*M*)	39 percent	41 percent
Inhibition (in vivo) by§		
Aminooxyacetic acid (10 mg/kg)	89 percent	100 percent
Ethanolamine *O*-sulfate (250 mg/kg)	62 percent	95 percent
Activity in crude tissue	167 nmole/min per gram of wet tissue	3.4 nmole/min per gram of platelets

*Extract partially purified from human cortex as described earlier (*3*). †For kinetic analysis, the GABA concentration was varied from 0.1 to 1 m*M* and the 2-oxoglutarate concentration from 0.04 to 0.4 m*M*. ‡Inhibitors were incubated with extract for 10 minutes at 37°C before addition of substrates. §Sprague-Dawley male rats (160 to 200 g) were given the indicated doses of aminooxyacetic acid or ethanolamine *O*-sulfate intraperitoneally in saline solution (two rats per dose). Controls were treated with saline solution. Rats were killed approximately 24 hours after dosing. The GABA aminotransferase activity was assayed in platelets and in whole brain homogenates from each rat.

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