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HEMOPERFUSION, RATE OF OXYGEN CONSUMPTION AND REDOX LEVELS OF MITOCHONDRIAL CYTOCHROME *c* ($+c_1$) IN LIVER IN SITU OF ANESTHETIZED RAT MEASURED BY REFLECTANCE SPECTROPHOTOMETRY

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Summary

Utilizing reflectance spectrophotometry, hemoperfusion, rate of oxygen consumption and redox level of mitochondrial cytochrome *c* ($+c_1$) in livers in situ of anesthetized rats were measured. The transition to the anoxic state was induced by raising the pressure on the liver surface to more than the hepatic blood pressure by pressing with the tip of the optical guide of the reflectance spectrophotometer. During this transition, the average oxygen saturation of hemoglobin in the liver in situ decreased linearly with time until it became 10—20% of the total. This was followed by reduction of mitochondrial cytochrome *c* ($+c_1$), which reached completion in 10—20 s. The measured O_2 consumption rate remained constant until the percentage of oxyhemoglobin in situ decreased to a critical level. There was then a decrease in the rate of O_2 consumption which was accompanied by a progressive reduction of cytochrome *c* ($+c_1$). It was shown that amounts of hemoglobin and mitochondrial respiratory chain cytochromes in the liver in situ could be measured non-invasively and could provide important signals for vital cellular functions. The changes in hemoperfusion and rate of O_2 consumption of the liver in situ following ethanol ingestion were also shown in rats, and are briefly discussed with respect to possible application of this method to study the pathophysiology of tissues.

Introduction

For non-destructive measurement of tissue oxidative functions, magnetic, polarographic and optical methods have been used. Organ reflectance or absorbance spectrophotometry has been applied to measure the redox changes of mitochondrial cytochromes [1—6], changes of local blood volume [4—6], oxy- and deoxyhemoglobin (or myoglobin) equilibrium [2,4—9], changes of catalase compounds [10] and chlorophyll formation [8] in intact organisms. These measurements, however, were usually limited to organs containing little or no hemoglobin, except in the case of determining the changes of local blood volume. In the previous paper, we demonstrated that visible-light reflectance spectrophotometry can measure qualitatively and semiquantitatively the changes of absorbance of hemoglobin and the respiratory chain components in intact gastric mucosa of anesthetized rats [11].

In this communication, we describe a method of measuring hemoperfusion, rate of O_2 consumption and redox level of cytochrome c ($+c_1$) in living livers in vivo and in situ from reflectance spectra. This was accomplished by measuring spectra sequentially following cessation of local blood flow by pressing the liver surface with the optical guide for reflectance spectrophotometry. The effect of ethanol ingestion on hepatic oxidative metabolism in vivo was also examined.

Materials and Methods

Animal. Non-fasting, male Wistar rats weighing 120—150 g were anesthetized with pentobarbital-sodium (35—40 mg/kg). The abdomen was opened through a mid-line incision. The flexible, light conducting quartz fiber bundle (6 mm in diamater, Sumitomo Electric Co. Ltd., Osaka) was guided through the incision and left in gentle contact with the median lobe of the liver, unless otherwise noted. Ethanol was administered to rats by gastric intubation at a dose of 1 g/kg body weight. 30% ethanol dissolved in water was given in 1 min.

Reflectance spectrophotometry. In vivo measurements of the average of oxy- and deoxyhemoglobin equilibrium, as well as absorbance of the intramitochondrial cytochromes were carried out by the fiber optics, which couple the liver surface to the spectrophotometer equipped with a computer and an image sensor as detector [12,13]. In this system, a reflectance (absorption) spectrum is obtained very quickly (0.01—0.64 s) and simultaneously at each wavelength in the region of 185 nm. Nine spectra which had been taken sequentially with variable intervals (0—5.76 s) were stored in a memory system. The computer is programmed to subtract a spectrum of standard material or of standard conditions from each spectrum. A randomized quartz fiber-glass optic bundle with diameter of 6 mm was used. The ratio of the numbers of fibers for reflected light to those for incident light was 1.5.

Spectral intensity as a function of hemoglobin concentration. In order to determine the relationship between the spectral intensity (absorbance and, partly, scattering) and the local hemoglobin concentration, reflectance spectra in anaerobic livers containing various concentrations of hemoglobin were taken (Fig. 1A). Hemoglobin concentrations in the liver were measured by a micro-method using low-temperature spectrophotometry [14,15]. The differ-

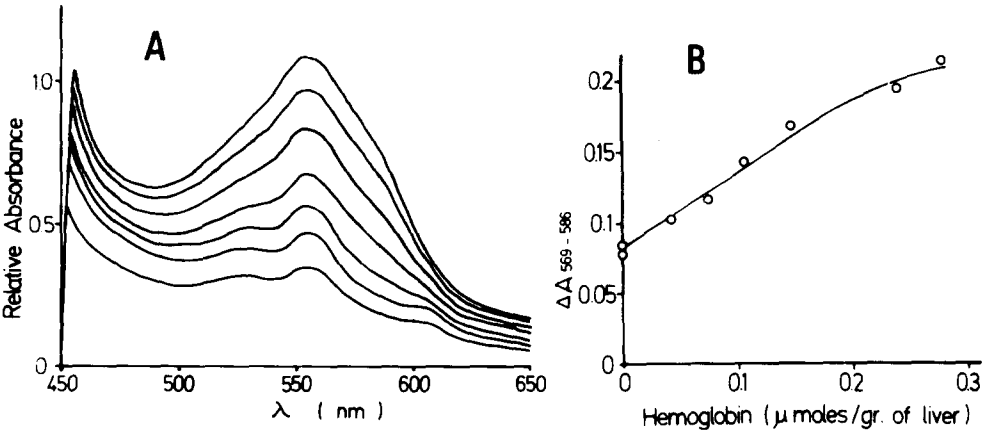

Fig. 1A. Reflectance spectra of anaerobic rat livers, which were perfused with Krebs-Ringer bicarbonate buffer containing various concentrations of blood hemoglobin. The spectra were taken using a randomized quartz fiber-glass optic. A reference spectrum of a standard material, white halon, was subtracted from each liver spectrum. B. Dependence of a function $\Delta A_{569-586}$ on the hemoglobin concentration in livers in situ.

ence in absorption between 569 and 586 nm (isosbestic points of oxy- and deoxyhemoglobin) was then plotted against the hemoglobin concentrations (Fig. 1B). The absorbance difference, $\Delta A_{569-586}$, under these conditions was found to be almost proportional to the concentration of hemoglobin in the liver. The value at zero hemoglobin concentration in the ordinate of Fig. 1B shows the absorbance of blood-free liver.

Determination of O_2-saturation of hemoglobin in the reflectance spectra. For calculating the percentage of O_2 saturation of hemoglobin from the in vivo reflectance spectrum (curve A in Fig. 2), we assumed that deoxyhemoglobin had a spectrum showing a linear increase of absorbance from 586 to 569 nm (curve B in Fig. 2). Consequently, subtraction of the difference in absorbance,

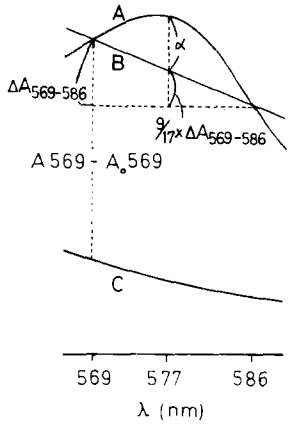

Fig. 2. A tentative method for calculating the O_2-saturation of hemoglobin from the reflectance spectrum. Curve A, a spectrum of a tissue (liver) in vivo; curve B, a line connecting the points of absorbances at 569 nm and 586 nm in the in vivo spectrum A; curve C, a spectrum of blood-free, perfused liver under aerobic conditions.

$9/17 \times \Delta A_{569-586}$, from the difference in absorbance between 577 and 586 nm, $\Delta A_{577-586}$, in the in vivo reflectance spectrum (curve A in Fig. 2) yields the oxyhemoglobin absorbance (value α). Hence, the O_2-saturation of hemoglobin, f, is calculated from the equation:

$$f = (K \cdot \alpha)/\Delta A_{569-586} \quad (1)$$

where the constant K (0.673) is calculated from the spectra of purified hemoglobin solution using the extinction coefficients presented by Assendelft [16]. For cancelling the absorbance of hepatic pigments, the absorbances at 569, 577 and 586 nm in the reflectance spectrum of blood-free, perfused liver from the same rat which had been used for the in vivo experiments (curve C in Fig. 2) were subtracted from the respective values in the in vivo spectrum.

Determination of the rate of O_2 consumption from the reflectance spectra. For determining the rate of O_2 consumption in the liver in situ, two reflectance spectra were taken when the liver was subjected to constant but different pressures. When such pressures were above the hepatic blood pressure, the result was complete blocking of blood flow in situ [13]. At the same time, this pressing caused an expulsion of part of the blood in the tissue in situ, establishing a new steady-state level of blood volume in situ within about 1 s after start of pressing [13]. Thus, during constant pressing, the O_2 would gradually be consumed by liver respiration, resulting in conversion from oxy- to deoxyhemoglobin with time. The approximate level of oxyhemoglobin after t s ($t >> 1$) pressing (Y) is calculated from the following equations;

$$Y_n = f_0(X_n - a) \quad (2)$$

$$Y_n = f_n X_n \quad (3)$$

where f_0 is the O_2 saturation of hemoglobin before pressing of the liver and f_n is that after t s pressing; X is the total hemoglobin in the blood in the pressed liver, calculated from the absorbance at 569 nm (A_{569}—$A_{0,569}$ in Fig. 2); a is the hemoglobin O_2 fraction that disappeared by liver respiration during t s

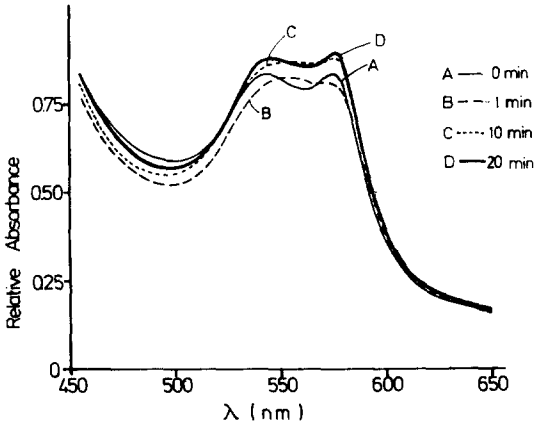

Fig. 3. Sequential changes in reflectance spectra of the liver of an anaesthetized rat following ethanol ingestion (1 g/kg). A, before ethanol ingestion; B, 1 min after the ethanol ingestion; C, 10 min after; and D, 20 min after the ethanol ingestion.

pressing. Thus, Δa is the rate of O_2 consumption by the liver, which is determined from two spectra obtained at different pressures from the following equation;

$$\Delta a = X_1 X_2 (f_1 - f_2)/(X_1 - X_2) \quad (4)$$

For measuring applied pressure, a device developed in our laboratory was used [17].

Perfusion of the liver. The liver was perfused via the portal vein. The perfusate was Krebs-Ringer bicarbonate buffer (pH 7.4) equilibrated with 95% O_2 and 5% CO_2 at 32°C; the flow rate was about 4.5 ml/g liver. Oxidation of mitochondrial cytochromes in perfused liver was effected by adding (20 μM) pentachlorophenol, and full reduction was obtained by replacing O_2 with N_2 gas.

Results

Reflectance spectra of rat liver in vivo

In the reflectance spectra of the liver of normal, anesthetized rats (Fig. 3A), two absorption maxima at 577 and 543 nm are shown, reflecting mainly oxyhemoglobin absorbance. The absorbance from cytochromes and other intracellular pigments should also contribute to these spectra, although under these conditions they apparently showed no appreciable bands. These spectra were reproducible, and the average hemoglobin concentration in the liver (probably several mm depth in the surface layer of the liver) in vivo of anesthetized rats was calculated using the calibration curve of Fig. 1B. It was about 0.20—0.30 μmol/g wet weight liver.

Curves B, C and D (Fig. 3) show the representative spectra after ethanol ingestion. Generally speaking, in several min after the ethanol ingestion (spectrum B), the absorbances at 577 nm somewhat decreased, and the absorption peak at 543 nm was red-shifted. The peak appeared at around 550 nm, where the cytochrome *c* of the mitochondrial respiratory chain has an absorption

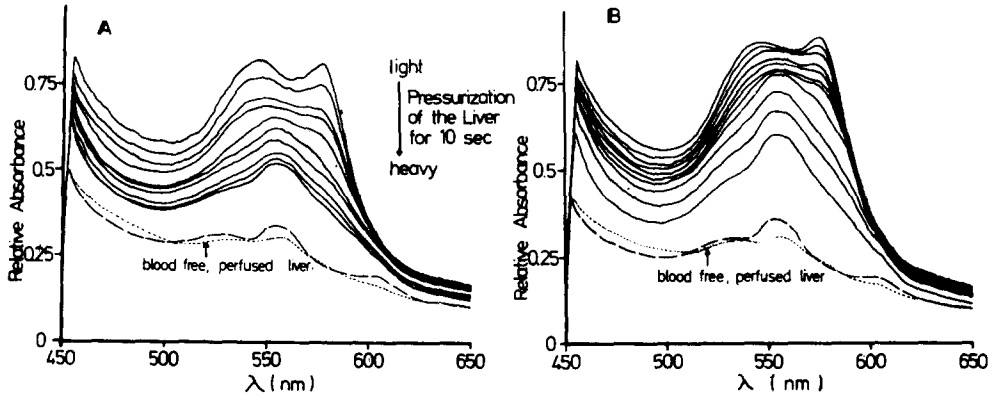

Fig. 4. Spectral changes of the livers following 10 s pressure on the liver in situ in control (A) and ethanol-treated (B) rats. The spectra were taken in 20—30 min after ethanol ingestion (1 g/kg). The dashed and dotted curves show the spectra of blood-free, anoxic and normoxic liver, respectively, from the same rats as in in vivo experiments.

maximum in its reduced form. These spectral changes suggest a conversion of some parts of oxyhemoglobin to deoxyhemoglobin and an increased degree of reduction of cytochrome *c* ($+c_1$).

Subsequently, about 10—20 min after ethanol ingestion, the local hepatic blood volume increased, as shown by an increased absorbance difference between 569 and 586 nm (Fig. 3C and D). This spectral pattern remained unchanged during 1—2 h observation. The hemoglobin concentration in the liver in situ at this time increased to more than 0.3 μmol/g liver. Except for this increase in spectral intensity, the spectrum (Fig. 3D) was quite similar to that of Fig. 3A obtained before ethanol ingestion.

Apparent O_2-saturation of hemoglobin in liver in vivo

The apparent O_2-saturation of hemoglobin in livers of normal, anesthetized rats (12 cases) was 63 ± 4% (mean ± S.D.). 1—5 min after ethanol ingestion it

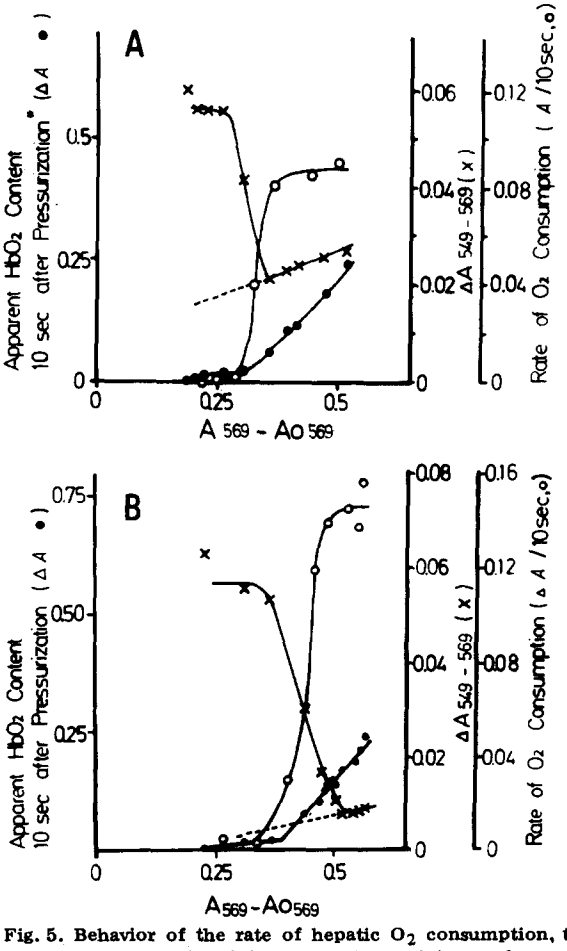

Fig. 5. Behavior of the rate of hepatic O_2 consumption, the apparent reduction level of cytochrome *c* ($+c_1$) ($\Delta A_{549-569}$) and the apparent, remaining oxyhemoglobin content 10 s after local pressing in livers in situ as a function of remaining local blood content ($A_{549} - A_{O,569}$) after 10 s local pressing of the liver. The values were calculated from Fig. 4. A, a control rat; and B, a rat 20—30 min after ethanol ingestion (1 g/kg).

was 55 ± 8%, which was significantly lower ($P < 0.05$, Student's t-test) than those obtained before ethanol ingestion. However, this lowered level of O_2 saturation returned to the control level usually within 10—20 min after ethanol ingestion.

Rate of O_2 consumption of living livers in situ

In order to measure the rate of O_2 consumption, the reflectance spectra of the liver were taken at an appropriate time after complete blocking of the blood flow in situ. For blocking of the blood flow, the tip of the optical guide was pushed to the liver at applied pressures (usually 200—400 g/cm^2 [17]) in excess of the hepatic blood pressure. Fig. 4 shows such spectra obtained following 10 s pressing of the liver in control and ethanol-treated rats, respectively.

The spectral patterns in Fig. 4A and B differ from each other, depending upon the difference in applied pressure. The lowest solid curves were obtained at a pressure of about 400 g/cm^2 for 10 s [17]. On the other hand, the dotted and dashed curves were obtained from isolated, blood-free livers perfused with Krebs-Ringer bicarbonate buffer (pH 7.4), which had been equilibrated with 95% O_2/5% CO_2 and with nitrogen, respectively. When the absorption bands at around 560—550 nm and 530—520 nm were compared between the lowest solid curves and dashed curves, (and also between the lowest solid curves of Fig. 4 and the curves of Fig. 1A), there was a strong similarity between them, which suggests that the lowest solid curves in Figs. 4A and B are characterized with absorbances of reduced intracellular cytochromes and also of deoxyhemoglobin. The absorption peaks and shoulders observed at around 560—550 nm and 530—520 nm may be due to reduced type b ($b_K + b_T$) and type c ($c + c_1$) cytochromes. Absorption bands of cytochromes b_5 and P-450 might also be partly involved. A slight shoulder at around 605 nm may be attributed to reduced cytochrome aa_3.

Pressing the liver in situ for a further 10 s induced essentially the same spectral pattern as in the lowest solid curves, which suggests that the cytochromes were then fully reduced and hemoglobin was completely deoxygenated. Thus, it was found that pressing caused blocking of the blood flow as well as expelling of some blood, in situ. It was demonstrated that when the blood supply had been blocked and approximately half of the blood in situ had been expelled by pressing (notice that the relative absorbance at 569 and 586 nm in the spectra of the lowest solid curves decreased to about half of the original control level), most of the oxygen in situ was consumed in approx. 10 s by liver respiration. These spectral changes were recovered usually within 1 min following release of pressure.

Fig. 4B shows the spectra of the pressed liver in a rat 20—60 min after ethanol ingestion (1 g/kg of body weight). Pressing caused changes of spectral pattern of ethanol-treated rat livers which were essentially the same as those of the control, but close observation of Fig. 4A and B reveals that the ethanol-treated rat had less O_2-saturation of hemoglobin than the control rat, when compared between the spectra with similar absorbances at 569 nm. This suggests an ethanol-induced stimulation of the rate of O_2 consumption.

Fig. 5 shows the relationship between the relative absorbance ($A_{569} - A_{O,569}$) and $f \cdot (A_{569} - A_{O,569})$. ($A_{569} - A_{O,569}$) is the absorbance difference

at 569 nm between the two spectra: one is the in vivo spectrum of Fig. 4, and the other is the in vitro, blood-free, normal spectrum (dotted curves of Fig. 4). Hence, $(A_{569} - A_{O,569})$ reflects the blood volume in the liver in situ. $f \cdot (A_{569} - A_{O,569})$ can be attributed to the remaining oxyhemoglobin content after 10 s pressing. A linear relationship was observed until the oxyhemoglobin content reached 10—20% of the total hemoglobin (Fig. 5). Thus, it seems that oxygen was consumed at a constant rate until the O_2 concentration became very low. Similar behavior was shown in the spectra in the case of ethanol-treated rats (Fig. 5B). The calculated rate of O_2 consumption from the above equations was also plotted in Figs. 5A and B. Ethanol ingestion indeed increased the rate of O_2 consumption in the liver in situ (0.16 ± 0.04 ΔA/10 s, whilst in control rats 0.12 ± 0.02 ΔA/10 s). The rate of O_2 consumption was constant until the apparent oxyhemoglobin content had fallen to approx. 10—20% of the total hemoglobin in the control rat (Fig. 5A), and to approx. 20—30% of the total hemoglobin in the ethanol-treated rat (Fig. 5B).

Behavior of the rate of O_2 consumption and the change of the apparent redox level of cytochrome c ($+c_1$)

In Fig. 5, the behaviors of the rate of O_2 consumption and of the apparent reduction level of cytochrome *c* ($+c_1$) in the livers in vivo under local pressing are also shown, and comparison was made between the control and the ethanol-treated rats. These were calculated from the spectra shown in Fig. 5. As 549 nm is close to the α maximum of reduced cytochrome *c* ($+c_1$) in the mitochondrial respiratory chain, and also is one of the isosbestic wavelengths of oxy- and deoxyhemoglobin, the absorbance difference between 549 and 569 nm reflects changes in blood volume as well as in the reduction level of cytochrome *c* ($+c_1$) (and partly of cytochromes b_5 and *P*-450).

With the decrease of $(A_{569} - A_{O,569})$, the $\Delta A_{549-569}$ diminished as shown in Fig. 5. However, with further decreases of $(A_{569} - A_{O569})$ the $\Delta A_{549-569}$ began to increase, and became full when the remaining oxyhemoglobin content $(f \cdot (A_{569} - A_{O,569}))$ reached 10—20% of the total hemoglobin. A sharp rise of $\Delta A_{549-569}$ would reflect an abrupt increase in the reduction level of cytochrome *c* ($+c_1$) in hepatocytes. The result suggests that the reduction level of cytochrome *c* ($+c_1$) remained essentially constant until the $\Delta A_{549-569}$ began to increase. After the calculated O_2 uptake became zero, a further small increase of absorbance at 549 nm was detected, concomitant with a further decrease in absorbance at around 577 nm.

Discussion

Aerobic organisms derive their energy mainly from aerobic metabolism and their cellular mechanisms are usually under the control of the respiratory rate. Cellular respiratory activity is so fundamental to maintaining cellular structure and function that under O_2 deficit the tissue will be damaged within a short time. Thus, measurement of tissue respiratory activity seems valuable for understanding of pathophysiology of organized tissues.

The present study reveals that the reflectance spectrum can measure qualitatively and quantitatively the changes of absorbance of hemoglobin, as well as

of the respiratory chain cytochromes, in livers in situ of anesthetized rats. Hemoglobin absorbance showed the local tissue blood volume and average O_2 content in circulating blood, whilst the oxidation-reduction levels of the respiratory chain components gave information as to intracellular O_2 sufficiency. Non-invasive measurement of these biochemical parameters is important for understanding the structure and function of tissues in vivo.

It was found that the rate of O_2 consumption in the liver in situ could be calculated by the conversion rate of a known concentration of oxyhemoglobin to deoxyhemoglobin during an appropriate time under complete blocking of blood supply in situ. For blocking of local blood flow, pressing of the liver surface in excess of the hepatic arterial pressure (100—130 mmHg), hepatic portal pressure (7—10 mmHg [18]) and the sinusoidal blood pressure (2—5 mmHg [18]) was effective [12,13]. For the pressing, pushing the tip of the optical guide onto the liver was very convenient because surface (Fresnel) reflectance, which was not informative for the inside metabolism of the tissue, could be avoided. Simultaneously, this pressing caused an expulsion of blood in situ from the tissue, establishing a new steady blood volume 1—2 s after pressing [12]. The extent of the blood expelled was dependent largely on the pressing force. The stronger the force, the more blood was expelled. Thus, pressing of tissues was useful also for controlling the tissue blood volume, i.e. the tissue hemoglobin O_2 content. Hence, we could measure a series of spectra of the liver which contained oxygen at normal to near zero levels. From such spectra, the hemoglobin concentration and its O_2-saturation level were determined using 569, 577 and 586 nm, at which the contribution of absorbances attributable to liver pigments were minimal during aerobic to anoxic transition [12,13].

During a rapid transition from a normal state to anoxia, the main components showing spectral changes at 549 and 569 nm were the cytochromes *c* ($+c_1$) as well as the hemoglobin [12,13]. Our in vivo analysis showed that as the O_2-saturation of hemoglobin was lowered, the degree of reduction of cytochrome *c* ($+c_1$) began to increase and reached almost 100% when the O_2-saturation of hemoglobin became less than 10% of the total of hemoglobin. This means that, as the intravascular O_2 concentration falls below a certain level (average hemoglobin O_2 percentage, 10—20%), there is a decrease in the rate of O_2 consumption by the liver, which is accompanied by a progressive reduction of cytochrome *c* ($+c_1$), a phenomenon which has been observed in isolated hepatocytes [19].

The rate of O_2 uptake in the liver in vivo of anesthetized rats seems to be rather fast. It was shown in the lowest solid curves of Fig. 4 that the respiratory cytochromes aa_3 and *c* ($+c_1$) were almost fully reduced after 10 s pressing when the local blood volume in the liver was expelled to approximately half: that is, the liver consumed O_2 in the blood in about 10 s. From studies of clamping the circulation, Chance et al. [20] have shown that the time from the start of fluorescence increase (360 nm excitation and 450 nm measurement) to half maximal fluorescence increase is 9 s for the liver of rats. If this fluorescence increase was attributable to NAD reduction in the respiratory chain, the value obtained by Chance et al. [20] would be similar to that obtained in the present study. Our results show that acute ethanol ingestion caused an increase in the rate of O_2 consumption in the livers in vivo of fed rats. Several investigators have

already reported an ethanol-stimulated O_2 uptake in liver tissue, as measured in slices or in perfused preparations [21—26]. Thus, our in vivo data confirm that ethanol ingestion induces a hypermetabolic state of the liver in rats [21,22].

The measured O_2-saturation of hemoglobin, 63% in the liver from fed rats, is well comparable with the hepatic mean PO_2 value (approx. 20—30 mmHg) obtained by a needle-shaped O_2 electrode [27], provided that the pH, pCO_2, temperature, and 2,3-diphosphoglycerate levels in the red blood cells in the liver are within normal range. The ethanol-induced reduction in the O_2-saturation of hemoglobin in the liver in situ (54%) would result from an increased rate of O_2 uptake. Further study is required for clarification of the mechanism by which the ethanol-induced O_2 uptake leads to hepatic injury, especially by chronic ethanol consumption.

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