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An improved method for the determination of protohaem in liver microsomes

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OMURA and Sato[1,2] determined the haem content of rabbit liver microsomes and of solubilized microsomal cytochrome, using a modification of the pyridine haemochromogen procedure of Paul, Theorell and Åkeson.[3] The modification consisted of using the difference spectrum (reduced–oxidized) of the pyridine haemochromogen instead of the absolute spectrum of the reduced form. On the basis of these determinations Omura and Sato calculated the molar extinction of cytochrome P-450 to be 91 cm^{-1} mM^{-1} for the increment (450–490 nm) in the CO difference spectrum and showed that they could account for all the microsomal haem as the sum of the cytochromes b_5 and P-450. This value of 91 cm^{-1} mM^{-1} has been used to calculate the cytochrome P-450 content of many materials other than rabbit liver microsomes, apparently without independent confirmation, although the corresponding cytochrome which is induced in the livers of rats treated with 3-methylcholanthrene appears to have a significantly higher molar extinction.[4] The balance of microsomal haem against total cytochromes b_5 and P-450 does not appear to have been confirmed either; indeed Bond and De Matteis[5] reported difficulty in achieving such a balance in rat liver microsomes. Similar difficulties were experienced in some of my experiments and this communication reports the location of the source of the discrepancy and how it can be overcome.

Rabbits were killed by air embolism and rats were killed by cervical dislocation. Livers were excised, cut into 1–2 mm pieces and rinsed three times with ice-cold 0·154 M KCl to remove as much blood as possible. The washed liver was homogenized with 0·154 M KCl (3 ml per gram liver) in a chilled homogenizer with Teflon pestle rotating at 600 rpm in a smooth-surfaced glass tube with 0·25 mm clearance. The use of KCl rather than sucrose in the homogenization medium minimizes the adsorption of haemoglobin.[6] The homogenate was centrifuged in a refrigerated centrifuge for 10 min at 10,000 *g* and the supernatant was centrifuged again for 1 hr at 100,000 *g*. The pellet was resuspended in 0·154 M KCl and centrifuged again for 1 hr at 100,000 *g*. The washed pellet was resuspended in 0·05 M potassium phosphate pH 7·4 to a volume of 1 ml for each gram of liver. An equal volume of 40% v/v glycerol in 0·05 M potassium phosphate pH 7·4 was then added to stabilize the cytochrome P-450.[7] This suspension of microsomes was stored at −20°.

The assays were carried out as described by Omura and Sato,[1] with modifications as described in the text where applicable. Spectra were traced with a Unicam SP800 recording spectrophotometer fitted with the SP850 scale expansion accessory, using cells of 1 cm light path.

Control experiments showed that the addition of glycerol and storage at −20° did not affect the results obtained.

The microsomes used in this investigation were effectively free from contamination with haemoglobin, as shown by the absence of a peak at 420 nm in the CO difference spectrum of reduced microsomes (Fig. 1).

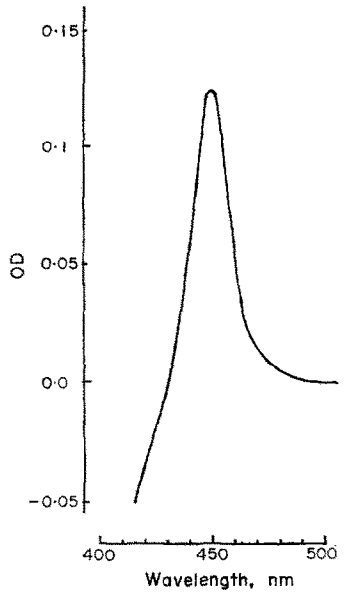

FIG. 1. CO difference spectrum of rat liver microsomes reduced with $Na_2S_2O_4$. Each ml of suspension contained microsomes from 0·083 g liver.

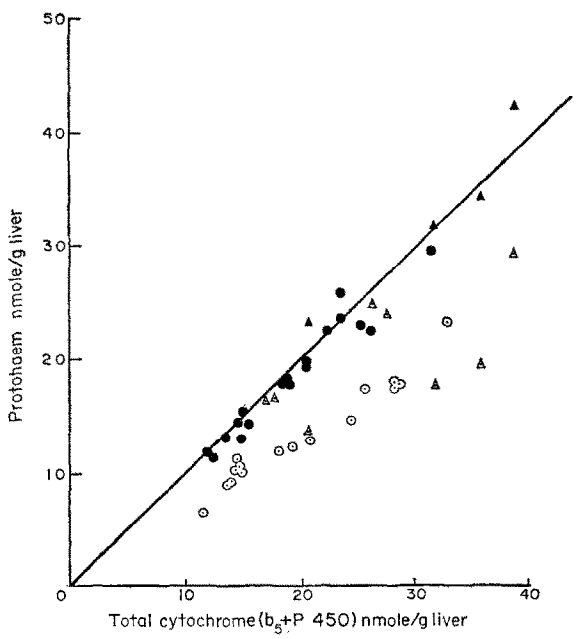

FIG. 2. Plot of apparent protohaem against total cytochrome in liver microsomes. ○● Rat liver microsomes. △▲ Rabbit liver microsomes. Open points show results obtained by the methods of Omura and Sato.[1] Filled points show results obtained when $3·3 \times 10^{-4}$ M $K_3Fe(CN)_6$ or 2mM H_2O_2 was added to the reference cell in the protohaem assay.

When rat liver microsomes were assayed by the methods of Omura and Sato,[1] the apparent total protohaem found was only about 70 per cent of the sum of the cytochromes b_5 and P-450 (Fig. 2, open circles). The reason for this was found to be the partially reduced state of the microsomes of the reference cell in the pyridine haemochromogen assay for protohaem. When microsomes were suspended in 20% pyridine, 0·1 N NaOH, a typical haemochromogen spectrum could be obtained either by reducing the microsomes in the test cell with sodium dithionite or by oxidizing them in the reference cell with potassium ferricyanide as suggested by Falk[8] (Fig. 3). This indicated that the microsomes, although prepared and assayed under aerobic conditions, were partially in the reduced state. The extent of this "spontaneous" reduction was relatively consistent in rat microsomes, usually between 20 and 40 per cent of the total. With rabbit microsomes values between 0 and 50 per cent reduction were found. The reason for this variation was not identified but it appeared to lie in the sample rather than in the reagents.

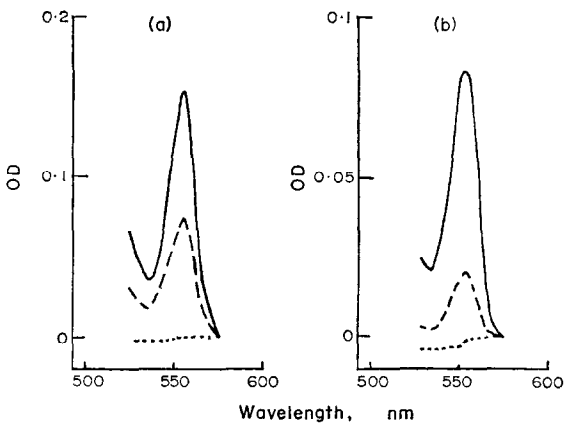

FIG. 3. Pyridine haemochromogen difference spectra of liver microsomes from (a) rabbit and (b) rat. ..... Base line with microsomes, pyridine and NaOH in both cells. – – – – Reference cell oxidized by $3·3 \times 10^{-3}$ M $K_3Fe(CN)_6$. ——— Reference cell oxidized by $3·3 \times 10^{-3}$ M $K_3Fe(CN)_6$ and test cell reduced by $Na_2S_2O_4$.

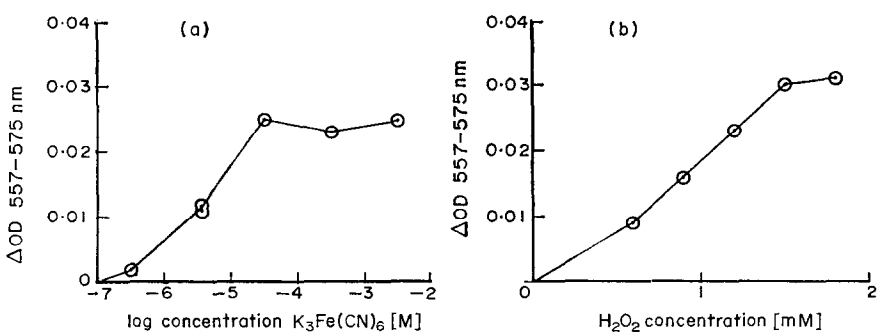

FIG. 4. Pyridine haemochromogen peak heights of rat liver microsomes with various concentrations of $K_3Fe(CN)_6$ or H_2O_2 in the reference cell. (a) $K_3Fe(CN)_6$, (b) H_2O_2. Each ml of suspension contained microsomes from 0·167 g of rat liver (different preparations in the two experiments).

Experiments in which increasing amounts of oxidant were added to the reference cell showed that the microsomes could be fully oxidized by 3×10^{-5} M $K_3Fe(CN)_6$ or by $1·5 \times 10^{-3}$ M H_2O_2 (Fig. 4).

When rat or rabbit liver microsomes were assayed for cytochrome P-450, cytochrome b_5 and protohaem as described by Omura and Sato[1] with the precaution of adding sufficient oxidant to the reference cell in the protohaem assay, good agreement was obtained between the estimates of protohaem and of total cytochrome (Fig. 2, filled points).

In some of the experiments with H_2O_2 the oxidant was added to the reference cell in the cytochrome b_5 assay also, but no evidence was found for spontaneous reduction of cytochrome b_5 under these conditions. Since the redox potential of cytochrome b_5[9] is higher than that of cytochrome P-450[10] and cytochrome P-450 is rapidly oxidized in the presence of O_2[1], the "spontaneous" reduction found in the protohaem assay is probably related to the specific conditions of that assay. However, the degree of reduction tended to vary between microsome preparations while the reagents used remained unchanged. It may be that microsomes can contain varying amounts of a reductant which only acts on the haem of the microsomal cytochromes at alkaline pH and/or in the presence of pyridine.

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Inhibition of 1-β-D-arabinofuranosyl cytosine phosphorylation in human livers by tetrahydrouridine*

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1-β-D-ARABINOFURANOSYL cytosine or ara-C (NSC-63878),† one of the most active antitumor agents, not only inhibits L1210 mouse leukemia and other transplantable rodent tumors,[1] but also induces remission in adult acute leukemia.[2] Ara-C is rapidly deaminated to the inactive product, 1-β-D-arabinofuranosyl uracil, ara-U.[3,4] A reduced pyrimidine nucleoside, tetrahydrouridine (THU), was found to be a potent inhibitor of the deamination of ara-C by enzyme preparations made from human livers or mouse kidneys.[5] Response to ara-C was related to the rate of its phosphorylation.[6,7] This report describes the phosphorylation of ara-C and the effect of THU on the phosphorylation with various tissues from man, hamster and mouse.

Liver samples were obtained at autopsy from persons who had died of cancer or after accidents. All other tissues were freshly collected from patients after surgery or from mouse or hamster. Chronic lymphocytic leukemia (CLL) cells were isolated from 60–100 ml of blood from patients with CLL.

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