THE TPNH-DEPENDENT OXIDATION OF LUMINOL CATALYZED BY RAT LIVER MICROSOMES

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Rat liver microsomes catalyze a TPNH-dependent O_2 uptake (Gillette et al., 1957). The enzymatic peroxidation of lipids and hydroxylation of various drugs are linked to this TPNH oxidase system of liver microsomes (Hochstein and Ernster, 1963; Gillette et al., 1957). Evidence has been obtained that TPNH-cytochrome c reductase (Horecker, 1950) is involved in lipid peroxidation, whereas both this flavoprotein and the CO-binding pigment (Klingenberg, 1958; Omura and Sato, 1964) participate in the hydroxylation of drugs (Orrenius et al., 1964a; Estabrook et al., 1963). Treatment of rats with phenobarbital in vivo results in a several-fold increase in the activities of oxidative demethylation and TPNH-cytochrome c reductase and in the amount of CO-binding pigment (Orrenius and Ernster, 1964b).

The participation of active reduction products from molecular oxygen in the TPNH-dependent microsomal hydroxylation reactions has been proposed by several investigators (Hayano, 1962). Whereas Mason advocates hydroxylation directly by a complex of enzymic ferrous iron and oxygen, $E\text{-}Fe^{++}O_2$, or by its reduction product, $E\text{-}Fe^{++}O$, (Mason, 1957), some authors postulate free radicals to be the hydroxylating molecular species. It has been suggested that hydroxyl or perhydroxyl radicals are generated by a terminal oxidase of the microsomes, which probably is identical with the CO-binding

pigment (Staudinger et al., 1964). Free radicals of this type, enzymatically generated, or formed by the decomposition of peroxides by a metal ion, have previously been found active in the chemiluminescent oxidation of luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) and related compounds (Totter et al., 1960; White, 1961). It has been suggested that the initial reaction step in the oxidation of luminol involves the transfer of an electron from the monoanion of luminol to a free radical, with the subsequent addition of molecular oxygen to the luminol radical formed (White, 1961).

The present report deals with the use of the luminol system in the study of the formation of "active" molecular species involved in the TPNH oxidase system.

Experimental. Microsomes were prepared according to Ernster et al. (1962). Measurements of oxygen consumption were started with microsomes and a TPNH-generating system (Orrenius and Ernster, 1964b). After addition of TPN, the TPNH oxidase activity was recorded. Aminopyrine was then added to a final concentration of 5 mM. The temperature was 30°C.

The apparatus used for the luminescence measurements is described elsewhere (Nilsson, 1964). A suspension of microsomes, ca. 6 mg protein, was injected into the reaction chamber containing 0.5 ml of a saturated solution of luminol in 1 M phosphate buffer, pH 8.0, and TPNH. The final volume was 2.5 ml. The luminescence was then recorded as a function of time under varying experimental conditions. The temperature was 22°C.

Rats were treated with phenobarbital as described earlier (Orrenius and Ernster, 1964b).

Results and Comments: When a microsomal suspension was injected into the reaction chamber containing the buffered luminol solution and TPNH, a luminescent oxidation of the hydrazide occurred. In Fig. 1

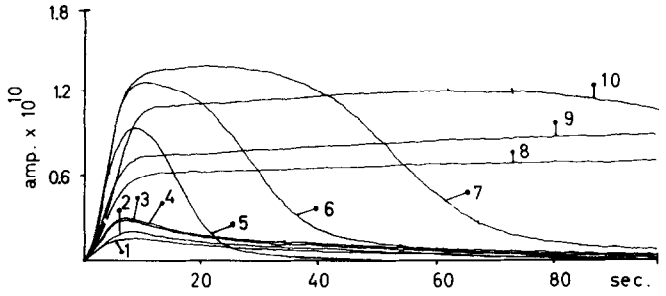

Fig. 1. Effect of TPNH and DPNH on the microsomal oxidation of luminol. Photocurrent from photomultiplier tube plotted against time. Concentrations of TPNH: 5) $1.4 \cdot 10^{-5}$M; 6) $2.8 \cdot 10^{-5}$M; 7) $5.6 \cdot 10^{-5}$M; 8) $11.2 \cdot 10^{-5}$M; 9) $16.8 \cdot 10^{-5}$M; 10) $28.0 \cdot 10^{-5}$M; Concentrations of DPNH: 2) $1.7 \cdot 10^{-5}$M; 4) $3.4 \cdot 10^{-5}$M; 3) $10.2 \cdot 10^{-5}$M; Curve 1 represents the oxidation of luminol in the absence of TPNH.

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the light intensity is recorded as a function of time at different concentrations of TPNH as well as of DPNH. The reaction is evidently highly specific for TPNH, and was found to be uneffected by dicumarol. Boiled microsomes were found completely ineffective. In the absence of TPNH or DPNH a low spontaneous oxidation of luminol was observed. Increasing concentrations of TPNH raised the maximal steady state reaction velocity to an optimum at a TPNH concentration of about $6 \cdot 10^{-5}$M. Above this value the steady state attained a lower, but more stable, level reflecting a longer duration of the reaction. This TPNH-dependent microsomal activity could also be measured by injecting a microsomal suspension, previously incubated with TPNH, into an alkaline solution of luminol containing hemin or cupric ions (Schales, 1939; Mayneord et al., 1955). In the latter case a very sharp luminescence peak of extremely short duration was observed. The enzyme system responsible for the oxidation of luminol could be rendered soluble by treatment of microsomes with Naja naja snake venom (Orrenius and Ernster, 1964c).

As in the case of the TPNH-dependent microsomal lipid peroxidation, the oxidation of luminol was greatly activated by the presence of ferric or ferrous iron, complexed by pyrophosphate, Fe-PP (Hochstein et al., 1964). Fig. 2 depicts the TPNH-dependent activated luminescence as a function of time at different concentrations of Fe-PP. Catalase had no effect on the reaction, and it is therefore unlikely that the enhancing effect of the iron on the luminescent reaction can be ascribed to an increased decomposition rate of free hydrogen peroxide resulting in the increased formation of radicals and oxygen.

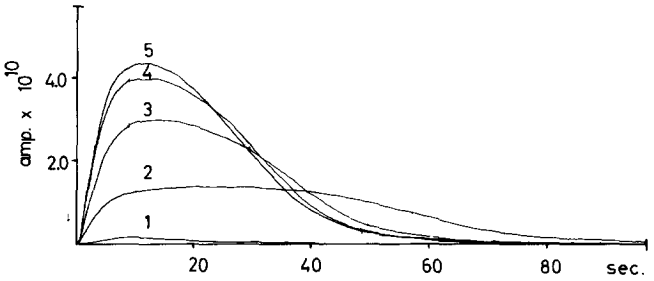

Fig. 2. Activation of the TPNH-dependent microsomal oxidation of luminol by complexed ferrous iron.
Photocurrent from photomultiplier tube plotted against time. Concentration of TPNH: $5.6 \cdot 10^{-5}$M. Concentrations of Fe-PP: 2) 0 M; 3) $3.6 \cdot 10^{-6}$M; 4) $7.2 \cdot 10^{-6}$M; 5) $21.6 \cdot 10^{-6}$M. Curve 1 represents the luminol oxidation observed in the absence of added TPNH at a Fe-PP concentration of $2.16 \cdot 10^{-5}$M.

Fig. 3 shows that addition of aminopyrine stimulated the TPNH-dependent O_2 uptake. This stimulation which equalled the amount of formaldehyde formed was increased several-fold when microsomes isolated from phenobarbital-treated rats were used. Incubation in the presence of CO completely abolished the aminopyrine-stimulated part of the O_2 uptake, whereas the original rate of oxygen consumption was not affected by CO.

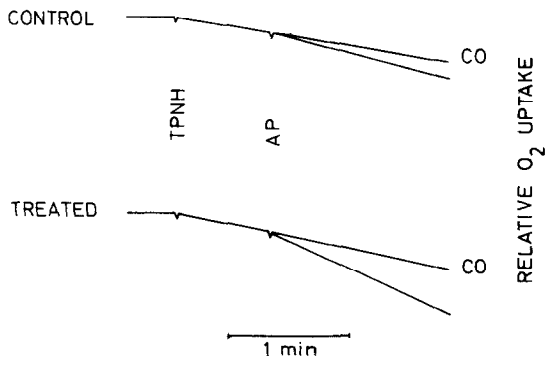

Fig. 3. Inhibition, by CO, of the aminopyrine-stimulated part of the TPNH-dependent relative O_2 consumption in microsomes from phenobarbital-treated rats and controls.

Before the measurements were started 4% O_2, 96% N_2 or 4% O_2, 56% N_2, 40% CO (marked CO) was bubbled through the system. Rats were given three phenobarbital injections. AP = aminopyrine.

Aminopyrine was shown to inhibit the luminol oxidation even in minute amounts. Fig.4 shows the effect of this drug at varying concentrations. The stimulation of the TPNH-dependent oxygen uptake caused by the addition of aminopyrine to a microsomal suspension, as well as formaldehyde formation, was found to be unaffected in the presence of luminol. Thus, the inhibition of the luminol oxidation observed in the presence of a drug may be explained on basis of competition between the luminol oxidizing system and the drug hydroxylating terminal oxidase for the reducing equivalents which, mediated by the TPNH-cytochrome c reductase, are required in the activation of molecular oxygen. In phenobarbital treated rats, where the level of this flavoprotein has been found to be markedly increased, the microsomal luminol oxidating activity was approximately doubled (Fig.5).

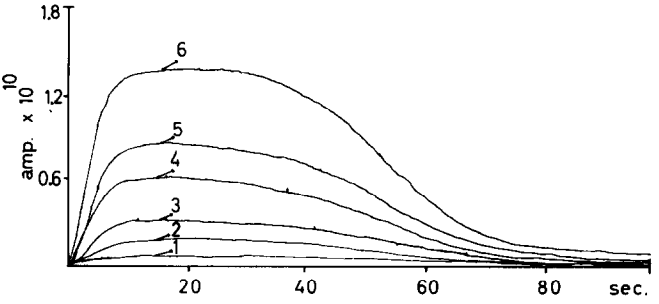

Fig. 4. Effect of aminopyrine on the TPNH-induced microsomal oxidation of luminol.

Photocurrent from photomultiplier tube plotted against time. Concentration of TPNH: $5.6 \cdot 10^{-5}$M. Concentrations of aminopyrine: 1) $8.0 \cdot 10^{-4}$M; 2) $2.0 \cdot 10^{-4}$M; 3) $0.8 \cdot 10^{-4}$M; 4) $0.4 \cdot 10^{-4}$M; 5) $0.2 \cdot 10^{-4}$M; 6) 0 M.

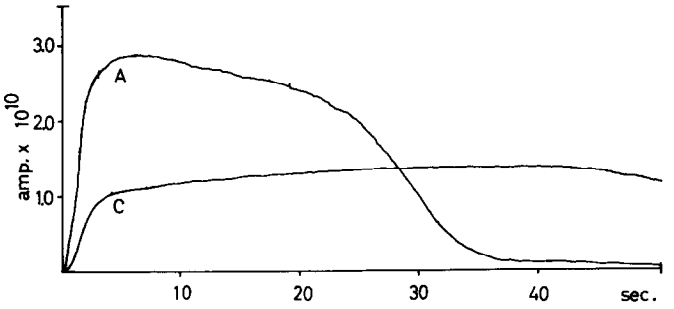

Fig. 5. Effect of phenobarbital treatment on the microsomal luminol oxidizing system.

Photocurrent from photomultiplier tube plotted against time. Concentration of TPNH: $5.6 \cdot 10^{-5}$M. Oxidative activity of liver-microsomes from phenobarbital treated rats, A, and of microsomes from controls, C.

From the results obtained it thus seems clear that in the absence of hydroxyl acceptor the addition of TPNH to microsomes induces the formation of an active intermediate, capable of reacting with luminol and probably also with unsaturated lipids. The intermediate is evidently not identical with free hydrogen peroxide but may be of free radical nature. The luminol reaction, similar to the

lipid peroxidation, does not seem to be inhibited by carbon monoxide, and the participation of the CO-binding pigment in the activation of molecular oxygen is not obligatory in these reactions.

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