THE CHEMICAL FORMATION OF EXCITED STATES DURING HYDROXYLATION OF THE CARCINOGENIC HYDROCARBON BENZO[α]PYRENE BY LIVER MICROSOMES[1]

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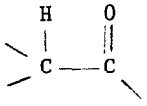
Summary: We have verified that the enzymatic hydroxylation of carcinogenic aromatic hydrocarbons produces product molecules in electronically excited states. Introduction of the carcinogen benzo[α]pyrene into liver microsomes from 3-methylcholanthrene-induced rats results in a significant chemiluminescence which is shown for the first time to be correlated with the enzymatic hydroxylation of the parent carcinogen. The kinetics of the chemiluminescence implicate the intermediate epoxide as the precursor to the excited state product molecules.

The inducible aryl hydrocarbon hydroxylase (AHH) system is required to convert carcinogenic polycyclic aromatic hydrocarbons (PAH) to reactive chemical species [1,2]. In 1975, Seliger [3] pointed out that the enzymatic hydroxylation of PAH's would yield chemical species containing the structures



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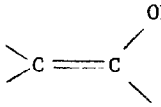

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which can be further oxidized spontaneously by molecular oxygen. A fraction of the parent carcinogens might therefore undergo a second oxygenation resulting in cleavage of an aromatic ring,

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Abbreviations: AHH, aryl hydrocarbon hydroxylase; PAH, polycyclic aromatic hydrocarbons; BP, benzopyrene; CL, chemiluminescence.

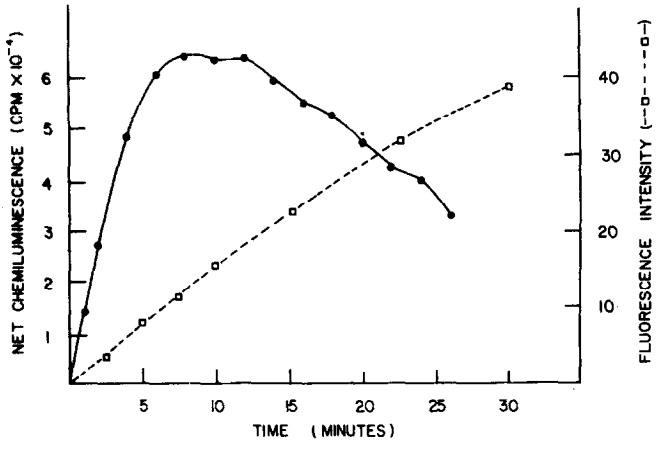
producing electronically excited carbonyl product molecules.

It was also suggested [4] that $n\pi^*$ excited state carbonyl products, because of their diradical properties, might be reactive carcinogenic forms of the parent carcinogens. Since a fraction of the excited state product molecules would fluoresce, light emission during enzymatic hydroxylation would be evidence for their formation. More specificically the kinetics of the predicted light emission should follow the kinetics of the enzyme reaction. Thus if we could demonstrate that the chemiluminescence of a carcinogenic PAH could be used as an assay for AHH activity, we would have unequivocal evidence that the production of excited states is due to the hydroxylation of the parent carcinogen.

We now report that the addition of the carcinogen benzo-[α]pyrene (BP) and the cofactor NADPH to AHH-induced liver microsomal extracts results in a significant chemiluminescence (CL). The maximum intensity of CL is directly proportional to the initial rate of hydroxylation of BP.

MATERIALS AND METHODS

Liver microsomes were isolated [5] from Long-Evans rats (Charles River Animal Farms) injected 24 hours previously with 3-methylcholanthrene (MC) (25 mg/Kg i.p. in 0.5 ml corn oil) or corn oil (0.5 ml) alone. The cytochrome c reductase-cytochrome P_{450} systems were isolated from MC-induced microsomal extracts [6]. Protein was determined by a modified Lowry procedure [7]. AHH activity was determined by adding BP and the cofactor NADPH and measuring the rate of increase of fluorescence of an alkaline extract of the reaction mixture [8]. Chemiluminescence intensities were measured in a modified light collecting system similar to that used for



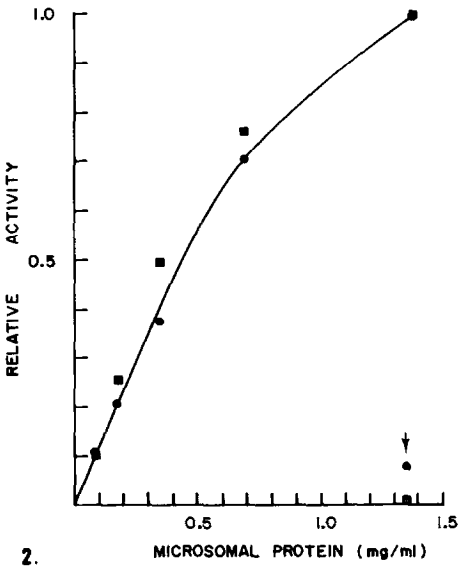


Figure 1. Kinetics of microsomal aryl hydrocarbon hydroxylase activity; chemiluminescence assay and fluorescence assay. For both assays the reaction mix contained 50 µmole Tris-HCl pH 7.5, 3 µmole $MgCl_2$, 1.35 mg microsomal protein isolated from liver of 3 methylcholanthrene induced rats, 75 nmoles benzo[α]pyrene and 0.5 µmole NADPH per milliliter at 22°C. The chemiluminescence intensity [●] was monitored continuously. The amount of hydroxylated products [□] was assayed by stopping the reaction at the indicated time with 1 ml of cold acetone and extracting the reaction mix into hexane. The hydroxylated products were then extracted from the hexane fraction into 1 N NaOH and the intensity of fluorescence was measured at 520 nm (excitation at 390 nm).

Figure 2. The dependence of the maximum chemiluminescence intensity and the rate of hydroxylated product formation on the concentration of microsomal protein isolated from the livers of 3-methylcholanthrene induced rats and non-induced rats. The reaction mix was the same as in Fig. 1, except the concentration of microsomal protein was varied. The maximum chemiluminescence intensity [circles] was taken from records of chemiluminescence intensity as a function of time (see Fig. 1) and the rate of hydroxylated product formation [squares] was the fluorescence intensity of alkali soluble products formed in 10 min. The solid symbols are for methylcholanthrene induced rats. The arrow indicates the maximum chemiluminescence intensity [●] and the initial rate of product formation [□] for microsomes isolated from non-induced rats at the minimum concentration for which a significant activity could be observed.

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liquid scintillation counting, using a specially selected low-noise phototube in a single photon counting mode.

Reactions were initiated by the addition of NADPH.

RESULTS AND DISCUSSION

The kinetics of the CL and the fluorescence of hydroxylated BP upon addition of BP and NADPH to rat liver microsomal extracts are shown in Fig. 1. The CL intensity increases to a maximum value at a finite time (ca. 10 min) after the initiation of the reaction with NADPH while the rate of formation of fluorescent alkali soluble product is a maximum initially and decreases to zero as one of the factors (usually NADPH) becomes limiting. The linear dependence upon microsomal protein concentration of both the maximum CL intensity and the initial rate of formation of 3-hydroxy BP are shown in Fig. 2 for microsomes from MC-treated rats and control rats injected with corn oil only. These same results have been measured for BP and NADPH added to the reconstituted AHH system [9] purified from MC-induced liver microsomes.

The addition of 7,8-benzoflavone (final conc. 100 nmole/ml), an inhibitor of the AHH system [10], reduced the observed CL intensity as well as the rate of BP hydroxylation by 90%. Addition of 1,2-epoxy 3,3,3-trichloropropane (final conc. 2 μmole/ml), an inhibitor of epoxide hydrase [11], increased the CL intensity by a factor of 1.3 and the rate of BP hydroxylation by a factor of 1.2.

The kinetics are consistent with the production of an epoxide intermediate in the hydroxylation of PAH's by AHH [12]. The epoxide hydrase reaction leading to the trans dihydrodiol has been proposed to compete with the spontaneous conversion of epoxides to phenols [13]. Inhibition of the epoxide hydrase system should increase the transient concentrations of epoxide and thereby of Form I intermediates,

resulting in an increase in the maximum CL intensity.

In order to explain the NIH shift an intermediate structure of the Form I is required between the epoxide produced by the AHH system and the phenol [13]. Form I is identical to the active site structure of essentially all bioluminescent substrates (luciferins) which evolved specifically to produce highly fluorescent $\pi\pi^*$ excited state product molecules. In other cases excited state ketone molecules produced by spontaneous oxidation of Form I result in singlet and triplet diradical states, the same as are produced photochemically [14] and can add covalently to ene bonds. In addition, chemical reactions involving H_2O_2 and chloride or hypochlorite ions have been suggested as sources of singlet oxygen [15] which can add to Form II [16], producing the same excited state products as from Form I. The mechanism described provides the chemical pathways for the original proposal by Anderson [17] that metabolic hydroxylation of carcinogenic PAH's should result in CL.

Only a small fraction of the total BP molecules undergoing hydroxylation undergo a second oxygenation by molecular oxygen to result in a detectable CL. Based on an absolute calibration of the photon detection system with the Luminol chemiluminescent reaction [18], the fraction of the total BP which undergoes a second oxidation to produce CL is approximately $\frac{10^{-8}}{\Phi}$ where Φ is the fluorescence yield of the excited state product molecule. The CL is therefore a tracer of the concentrations of epoxide intermediates formed as the result of the rate of oxygenation of the parent carcinogen by the AHH system.

This report demonstrates only the production of potentially

reactive excited states during enzymatic hydroxylation of carcinogenic PAH's. We propose to examine separately their possible reactivity, i.e. mutagenicity in bacterial systems [19].

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