ELECTRON SPIN RESONANCE AND OPTICAL SPECTRA OF THE COMPLEXES OF DIALKYLNITROSOAMINES WITH CYTOCHROME P-450[1]

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Summary: Optical absorpiton and Electron Spin Resonance studies have been conducted on cytochrome P-450 complexes with dialkylnitrosoamines. Under reducing conditions, it was observed that these labile carcinogens when incubated with cytochrome P-450 either derived from rat liver microsomes or as purified preparations from rabbit liver or camphor, gave optical and ESR spectra characteristic of cytochrome P-450-NO complexes. Verification of the spectra was made by comparison with complexes derived either from reduced $NaNo_2$ or NO gas. These results suggest that the dialkylnitrosoamines are metabolized by the mixed function oxidase system in microsomes to yield free NO which complexes to cytochrome P-450 to rapidly yield cytochrome P-420.

1. Introduction

It is well known that the dialkylnitrosoamines require biological activation before they are able to exert their hepatoxic and carcinogenic effects. These compounds are generally thought to be metabolized by the liver to yield initially unstable monoalkylnitrosoamines. This is followed by further decomposition to reactive diazoalkanes or carbonium ions, which act as alkylating agents yielding finally formaldehyde and then CO_2 (1-3). A suggested reaction scheme would be:

$$(CH_3)_2\ N\text{-}N = O \rightarrow CH_3\ \overset{O}{\overset{\|}{N}}\text{-}CH_2\text{-}OH \rightarrow CH_3\text{-}N = N\text{-}OH + CH_2O$$

$$\downarrow\ H^+, O_2$$

$$CH_3^+ + N_2 + H_2O + CO_2$$

There appears to be ample evidence to suggest that the actual metabolism of the dialkylnitrosoamines requires the microsomal mixed function oxidase system. However, there is very little data on the direct interaction of dialkylnitroso-

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DMNA - Dimethylamine; DENA - Diethylnitrosoamine; ESR - Electron Spin Resonance

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amines or their metabolic intermediates with cytochrome P-450. Iose and Slater (4), however, suggested that dimethylnitrosoamine can form complexes with cytochrome P-450. Their results could not be confirmed by D'Acosta e. al. (5).

In the results presented here, we have used ESR and spectrophotometric studies to probe the interaction of dimethylnitrosoamine (DMNA) and diethylnitrosoamine (DENA) and some of their metabolic intermediates with cytochrome P-450. The source of P-450 being from intact rat liver microsomes, or as purified preparations from rabbit liver and camphor. Out studies suggest that a principal product in the metabolism of these compounds is free NO which rapidly forms a liganed complex with cytochrome P-450.

2. Materials and Methods

Microsomes were prepared from Wistar rat liver in 0.05M tris buffer (pH 7.4) containing 1.15% KCl as described by Levin et. al. (6). Cytochrome P-450 in microsomes was determined by the method of Omura and Sato (7). Protein was determined by the method of Lowry et. al. (8). ESR spectra were obtained on a Varian E-4 Spectrometer at 77°K. Optical spectra were recorded on a Beckman Dual wave length spectrophotometer (Model No. 25). Purified cytochrome P-450 from rabbit liver and cytochrome P-450 from camphor were obtained from the laboratory of Dr. Kerry Yasunobu.

DMNA and DENA were obtained as reagent grade from Eastman Kodak and were used as is.

3. Results and Discussion

The difference spectra of both DMNA and DENA (40-80mM) incubated with rat liver microsomal cytochrome P-450 show Soret band maximum at 420 nm (Fig. 1, dashed curve).

Using sodium dithionite reduced microsomes, both DMNA and DENA show significant absorptions at 450-453 nm with an additional absorption partially resolved at 482 nm and at 585 nm (Fig. 1, solid curve).

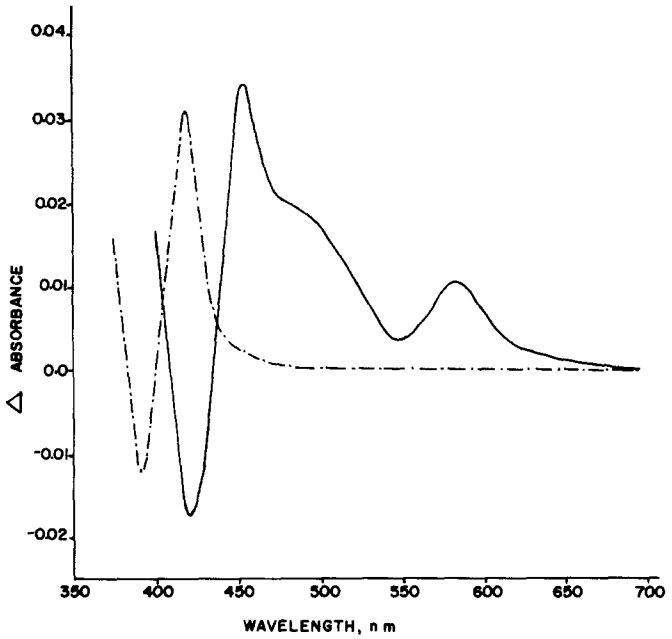

Fig. 1. Difference spectra of cytochrome P-450 in microsomes complexed with dimethylnitrosoamine (DMNA) or diethylnitrosoamine (DENA). The dashed curve is the oxidized form and the solid curve, the reduced form using sodium dithionite.

ESR studies at 77°K of ferric cytochrome P-450 dialkylnitrosoamine complexes show no changes in the typical low spin signals characteristic of cytochrome P-450 (g = 2.43, 2.26 and 1.91, Fig. 2, dashed curve). In contrast, however, the ferrous complexes of cytochrome P-450 in the presence of both DMNA and DENA revealed an intense ESR signal with triplet hyperfine splittings (Fig. 2, curve 2) which are characteristic of NO-complexes of the hemoproteins (9-13), including cytochrome P-450 (11, 14).

These results support the assumption that DMNA and DENA are metabolized to yield free NO in the presence of reduced microsomes and the optical spectra of these ferrous microsomal cytochrome P-450-carcinogen complexes are due to cytochrome P-450-NO complexes. In order to confirm this assumption, similar experiments were carried out using $NaNO_2$ in place of the alkylnitrosoamines. It is well known that $NaNO_2$, under reduced conditions, yields free NO (9,10,12-14). Virtually identical optical and ESR spectra of the reduced microsomal ferrous

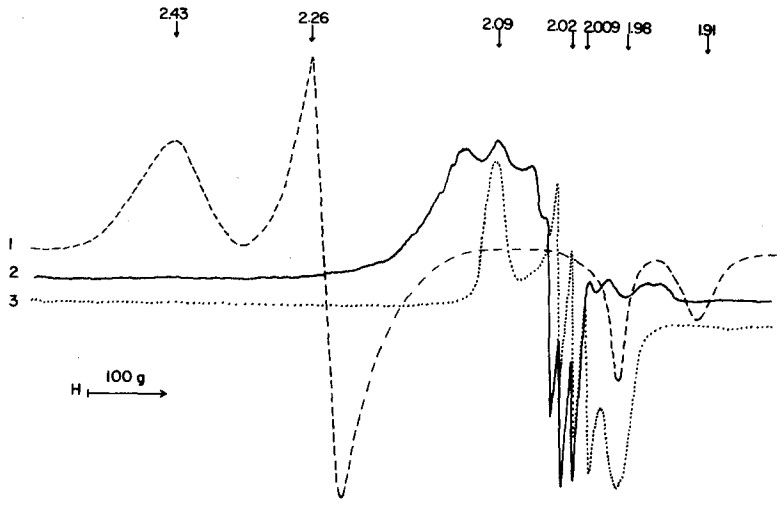

Fig. 2. ESR spectra at 77°K of purified preparations of cytochrome P-450$_{CAM}$ from camphor stabilized with substrate. Curve 1 is the oxidized form low spin. Curve 2 is the reduced form complexed with DMNA or DENA. Curve 3 is the reduced form complexed with NO added as the gas or from reduced NO_2^-.

cytochrome P-450 were observed using $NaNO_2$ in place of the nitrosoamines (See Fig. 3).

If the identification of a P-450-NO complex is correct, we would expect similar results in studies of the interaction of DMNA and DENA with reduced purified preparations of cytochrome P-450$_{LM}$ from rabbit liver. In fact, we did observe ESR signals with triplet splittings similar to those observed with microsomes in the reduction by sodium dithionite of purified preparations of cytochrome P-450$_{LM}$ in the presence of DMNA and DENA or $NaNO_2$.

The optical spectra under these conditions, however, did not show the absorption maximum of 450-453 nm as was the case observed with microsomes.

Ferrous cytochrome P-450$_{LM}$ purified in the presence of either carcinogens or $NaNO_2$ shows instead a maximum at 443 nm (the corresponding Ferric form has an absorption at 425 nm). The same results were obtained with purified preparations of cytochrome P-450$_{CAM}$. It should be pointed out that similar absorptions were observed by Ebel et. al. for Ferrous cytochrome P-450 saturated with NO gas (14).

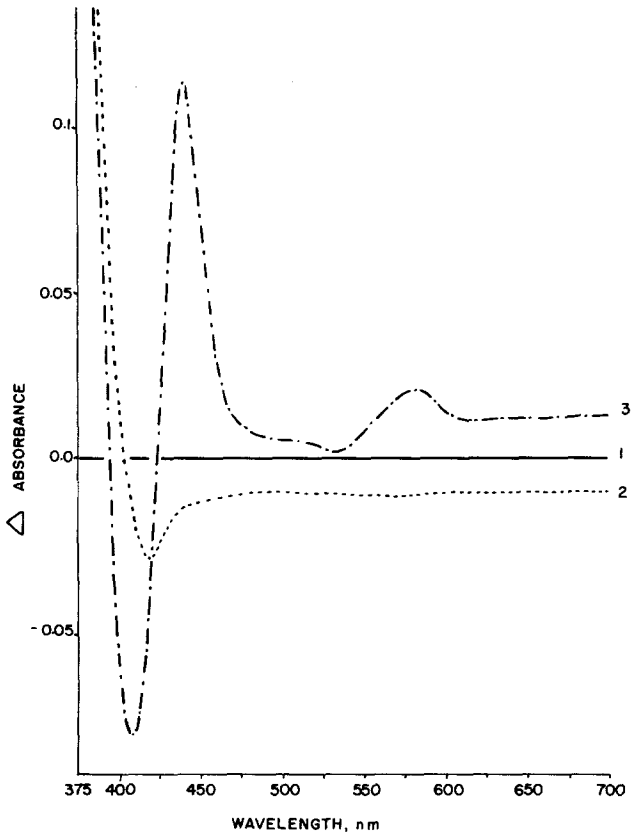

Fig. 3. Difference spectra of cytochrome P-450$_{CAM}$ with substrate.
Curve 1 is the base line.
Curve 2 is with $NaNO_2$ added to the sample cuvette.
Curve 3 is with dithionite added to both sample and reference cuvettes.

A possible interpretation of our results and those of the literature (9-14) would be that under reduced conditions, both DMNA and DENA decompose to yield free NO as one of the intermediates which then becomes liganed to cytochrome P-450. However, these results do not explain the appearance of the unusual absorpiton at 450-453 nm in the interaction of DMNA and DENA with rat liver microsomes.(See Fig. 4, curve 2). As we have shown in Fig. 4, the actual NO-Ferrous cytochrome P-450 complexes show absorptions at around 440-443 nm (Fig. 4, curve). One possible explanation for this absorption at 450-453 in microsomes may come from an analyses of the results of Franklin et. al. (15-17). In their

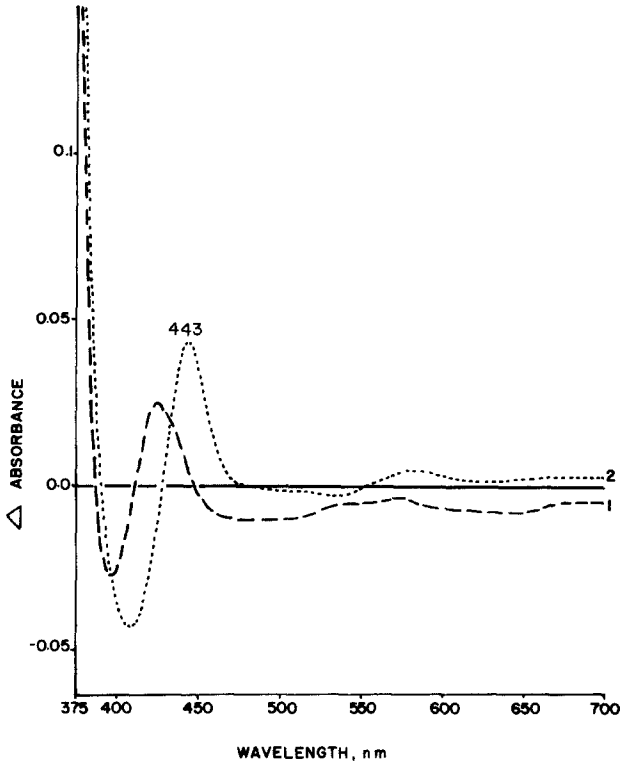

Fig. 4. Difference spectra of cytochrome P-450$_{CAM}$ with substrate.
Curve 1 is with DENA added to the sample cuvette.
Curve 2 is with dithionite added to both the sample and reference cuvettes.

work, they had shown that certain amines (amphetamine, benzphetamine, etc.) can form complexes with reduced liver cytochrome P-450 which yield absorptions at 455 nm. In accordance with this data, we can assume that the dialkylnitrosoamines which are studied here could possibly also form complexes with Ferrous cytochrome P-450. Probably, it is possible that such complexes are not stable (as indicated in references 15-17) and can transfer into 450 nm-absorption complexes from Ferrous cytochrome P-450: P-450-NO (443 nm, triplet ESR signal) and P-450-dialkylnitrosoamine itself (probably 455-453 nm, NO ESR triplet signal) can lead to an overlapping of absorption maxima yielding as a result, the absorption observed at around 450 nm.

Supporting this interpretation, one can see from Fig. 1 in the spectra of

reduced cytochrome P-450, there is an unresolved maximum, which perhaps is reflected in the existence of an actual maximum at 455 nm. Secondly, in some cases, in our studies, we observed spectra in which the maximum was shifted to the 443-448 nm region (probably due to a higher ratio of P-450-NO complexes), although the average statistical absorption was around 450-453 nm.

We have no explanation as to why $NaNO_2$ which very rapidly forms NO under reduced conditions shows the same absorption at 450-453 nm.

The additional formation of CO during the interaction of the dialkylnitrosoamines or $NaNO_2$ with Ferrous microsomal cytochrome P-450 could explain these results, but we do not have any evidence for it.

The formation of NO from dialkylnitrosoamines and subsequent formation of Ferrous cytochrome P-450-NO complexes led to significant alterations of the cytochrome P-450. As was shown by Ebel et. al. (14), the complex Ferrous cytochrome P-450-NO very quickly transfers to a cytochrome P-420-NO complex which is characterized by broad ESR lines at g~2.06-2.09. In our experiments with rat liver microsomes or purified cytochrome $P\text{-}450_{LM}$ from rabbits liver, we observed mostly this P-420-NO complex. Only complexes of Ferrous cytochrome $P\text{-}450_{CAM}$ with NO (generated by reduction of $NaNO_2$) gave ESR spectra corresponding to the native form of cytochrome P-450-NO (14) (Fig. 2, curve 3). The presence of substrate (camphor) in this system probably promotes the stabilization of the complexes formed. These ESR signals are characterized by two distinct components at g = 2.09 and g = 1.98. In addition, there is a slight shift in g-value for the triplet hyperfine splitting in the region of high field compared with the cytochrome P-420-NO complexes.

Thus, we can conclude that DMNA and DENA strong carcinogens can lead to the conversion of cytochrome P-450 to cytochrome P-420.

There is a considerable reason to suggest that this conversion is due initially to the interaction of NO, which is a product in the metabolic decomposition of these carcinogens under reduced conditions, with native cytochrome P-450.

An alternate explanation of the origin of the NO complex formed in the microsomal system with the nitrosoamines could be due to direct reduction of the

amines by dithionite to produce NO in the optical experiment to obtain reduced cytochrome P-450. In principal, this cannot be excluded, however, using a weaker reducing agent such as ascobic acid (10^{-3}M) produced the same results suggesting that the NO is probably the result of microsomal decomposition. Further support of this was observed by us in spin-trapping studies in this system where under induced lipid peroxidation conditions a labile free radical of the R_2N type is formed in microsomes incubated with these nitrosoamines (18).

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