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Interaction of Dimethylformamide and Its Metabolites with Cytochrome P-450 in Microsomal Fraction of Rat Liver

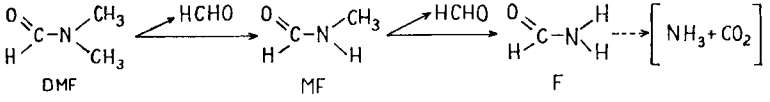
Interakcja dwumetyloformamidu i jego metabolitów z cytochromem P-450 w mikrosomalnej frakcji wątroby szczura

Взаимодействие диметилоформамида и его метаболитов с цитохромом P-450 в микросомальной фракции печени крысы

Dimethylformamide (DMF) is widely used as an organic compound solvent, especially in plastic industry. However, it displays certain toxic action. It is easily absorbed into respiratory tracts, alimentary duct and uninjured skin and thus it can cause injury of parenchymatous organs and ailments of alimentary duct (3, 4, 8, 9, 12).

It has been proved in experimental investigations (2, 5, 6) that metabolism DMF consists in gradual demethylation, proceeding probably according to a scheme presented below:

Suggested scheme of DMF biotransformation *in vivo* after Kimmerlle and Eben (5)



The intermediate products of the occurring reactions are methylformamide (MF) and formamide (F). The latter product of conversion undergoes probably further degradation into NH_3 and CO_2.

It is commonly known that in numerous reactions of xenobiotics biotransformation there participates a microsomal mixed-function oxi-

dase system catalyzing also some dealkylation reactions. An intermediate stage of these reactions is the binding of substrate with cytochrome P-450, and its consequence — a modification of absorption spectrum of cytochrome P-450 (13). It is also known that the type of cytochrome P-450 spectrum is determined, among others, by the kind of the added substrate and the nature of a bond (13).

The paper aims at determining the type of difference spectra for DMF and its metabolites: MF and F.

MATERIAL AND METHODS

Animals. The experiments were carried out on male rats of Wistar breed, weight 150—250 g. The animals were fed with granulated laboratory fodder LSM and they were given drinking water *ad libitum*. 7 days before the experiment drinking water has been completed with 0.1% additions of phenobarbital in order to induce the enzymes of microsomal fraction.

Preparation of microsomes. The rats were killed through decapitation, the livers were isolated, washed 3 times with cold 1.15% KCl solution, dried on the blotting-paper, and after weighing they were homogenized with 3 volumes of buffer, containing: 20 mM Tris-HCl, 150 mM KCl and 1 mM EDTA, of *pH* 7.4. The homogenate was rotated for 20 min. at 9000×g, obtaining postmitochondrial fraction. Microsomes were isolated after Baker and co-workers (1), solving the obtained fraction with 12.5 mM saccharose solution, containing 8.8 mM $CaCl_2$, in the ratio 1 : 5. In order to standardize the investigations there have been prepared microsomal fraction preparations of protein concentration 2 mg/ml.

Determination of difference spectra. The concentration of cytochrome P-450 and cytochrome P-420 in microsomal fraction have been determined by means of Omura and Sato's method (1).

In order to determine difference spectra of the complex: cytochrome P-450 (Fe^{+++})-substrate, 3 ml of microsomal fraction were added to each of two cuvettes, then substrate was introduced into the investigated sample; to the control sample the appropriate amount of buffer was added. Absorbance has been registered on the dual-path Beckman spectrophotometr, type Acta C-III.

To show the effect of the examined compounds (DMF, MF and F) on the complex: cytochrome P-450 (Fe^{++})-CO, 2.5 ml of microsomal fraction and some crystals of sodium dithionite were added to two cuvettes and next, gaseous carbon monooxide was bumbled through the examined sample for 3 min. Having introduced the appropriate substrate into the examined sample, and the equivalent amount of buffer — into the control sample, the difference spectra were registered on Beckman spectrophotometer, Acta C-III, as above.

The concentration of protein was determined after Lowry and co-workers' method (7) with crystalline bovine serum albumin, as standard. Dimethylformamide (DMF), methylformamide (MF), and formamide (F) from Reachim firm (Soviet Union) were used in the experiments.

RESULTS

The difference spectra of oxidized cytochrome P-450, present in microsomal fraction induced by phenobarbital were showed in Fig. 1a—c in the presence of changeable concentrations of DMF, MF and F.

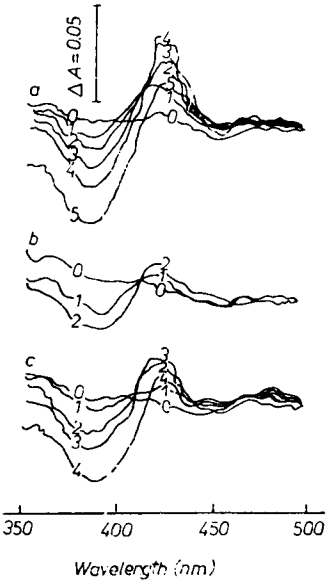


Fig. 1. Difference spectra of the complex: cytochrome P-450 (Fe^{+++})-microsomal fraction substrate of the liver of rats induced with phenobarbital in the presence of the investigated compounds: a — DMF in concentrations: 1 — 0.104 M, 2 — 0.26 M, 3 — 0.5 M, 4 — 1.4 M, 5 — 2.52 M; b — MF in concentrations: 1 — 1.82 M, 2 — 3.3 M; c — F in concentrations: 1 — 0.16 M, 2 — 1.12 M, 3 — 2.26 M, 4 — 4.1 M

It has been proved that all the examined compounds cause the change of cytochrome P-450 spectrum with the strongly marked maximum in the range 417—418 nm and the minimum — in the range 385—392 nm.

The analysis of absorbance changes depending on the concentration of the substrates introduced into the reaction mixture shows that DMF acts most strongly, causing the appearing of a distinct spectrum already at the concentrations ca 50 mM. A slightly weaker action is that of F (ca 80 mM), whereas in the case of MF the difference spectrum occurs just at many times higher concentrations, exceeding 1.5 M.

It is worth-while noting that in the case of DMF and F an increase of concentration respectively over 1.4 and 2.2 M (the curves 5 and 4 in Figs. 1a, c) causes the weakening of intensity of difference spectra which is probably connected with destruction of cytochrome P-450.

Table 1 presents the effect of increasing concentrations of DMF, MF and F on the complex: reduced cytochrome P-450-CO. It is expressed by changes in the concentration of cytochrome P-450 and occurrence of cytochrome P-420, at respectively high concentrations of the substrates.

Table 1. Effect of variable concentrations of DMS, MF and L on the complex: cytochrome P-450 (Fe^{++}) — CO in microsomes of rats liver treated with phenobarbital *

Volume of added substrate l	DMF			MF			F		
	Conc. M	P-450 nmole/ /mg protein	P-420 nmole/ /mg protein	Conc. M	P-450 nmole/mg protein	P-420 nmole/mg protein	Conc. M	P-450 nmole/mg protein	P-420 nmole/mg protein
-	-	1.950	-	-	1.885	-	-	1.951	-
10	0.05	1.945	-	0.07	1.864	-	0.08	1.914	-
50	0.26	1.933	-	0.33	1.820	-	0.41	1.888	-
100	0.50	1.846	-	0.65	1.772	-	0.81	1.840	-
200	0.96	1.727	-	1.26	1.721	-	1.56	1.760	-
300	1.40	1.612	-	1.82	1.661	-	2.26	1.674	-
600	2.52	1.029	0.17	3.30	1.451	-	4.10	1.390	-
900	3.45	0.396	0.72	4.50	1.046	0.19	5.60	0.810	0.54

* When enumerating concentrations of cytochrome P-450 and cytochrome P-420 there was taken into consideration the dilution of microsomal fraction with substrates; the difference spectra has been determined within the time not longer than 6 min. from the moment of adding the substrates.

Figure 2 illustrated the change in the concentration of cytochrome P-450 (Fe^{++})-CO by the analyzed compounds. The curves presented on the system of co-ordinates show that DMF is most related to iron (Fe^{++}) of cytochrome P-450, whereas MF and F are less related, respectively

Figure 3 presents the formation of non-active form of cytochrome P-450, i.e. cytochrome P-420. It was observed that at the examined

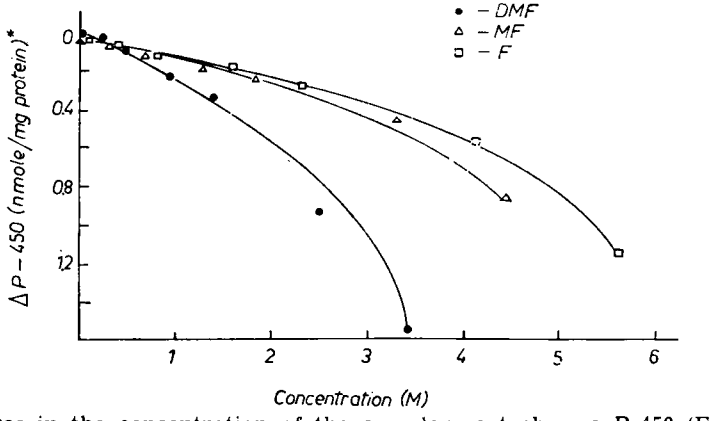


Fig. 2. Changes in the concentration of the complex: cytochrome P-450 (Fe^{++})-CO under the influence of variable concentrations of DMF, MF and F. * Concentration of cytochrome P-450 in microsomes not treated with substrates minus concentration of cytochrome P-450 in microsomes treated with substrates

compounds the concentrations exceeding, respectively: 1.4 M for DMF, 1.8 M for MF and 2.2 M for F there appear the distinct absorbance maximum at 420 nm, and two additional, much weaker maxima, at 540 and 570 nm.

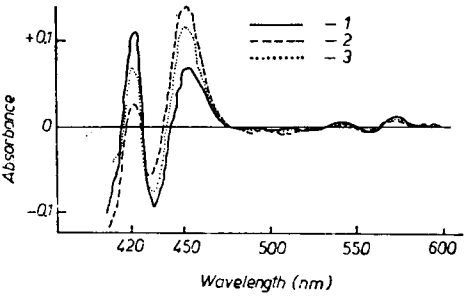


Fig. 3. Difference spectra of the complex of reduced cytochrome P-450-CO of microsomal fraction of liver induced with phenobarbital in the presence of final concentrations of substrates; 1 — DMF (3.45 M), 2 — MF (4.5M), 3 — F (5.6 M)

DISCUSSION

The change of cytochrome P-450 of type I with the maximum in the range 385—390 nm and the minimum ca 420 nm; of type II, with the maximum in the range 425—435 nm and the minimum — 390—405 nm as well as the reversal type I with the maximum at 420 nm and the minimum at 388—390 nm, is considered to be the consequence of binding of substrates with this enzyme (13). The difference spectra of type II is characteristic of the compounds being strong ligands and able to dislodge CO from the complex with a reduced form of cytochrome P-450 (13, 14). Substances containing nitrogen in the molecule, including also the substituted amine groups, e.g. aniline, nicotinamide (12, 13) belong to this type of compounds.

As it results from our investigations, dimethylformamide and its metabolites modify the cytochrome P-450 spectrum in a way approximate to the typical nitrogen ligands. The minimum of absorbance of the investigated compounds lies within the range characteristics of difference spectra of type II, whereas the maximum of absorbance undergoes a slight shift towards shorter waves. Though the shift of this type could suggest a difference spectra of the reversal type I, however, the chemical structure of the studied compounds and dislodging of carbon monooxide from the binding with cytochrome P-450 would rather prove the share of strong ligand in the cytochrome bond with iron. According to T s u c h i d's (10) classification, oxygen ligands (e.g. R-OH, H_2O) are characterized by considerably weaker strength of ligand field than the ligands containing nitrogen (e.g. NH_3, ethylenodiamines) and, thereby,

a weaker bond with iron. Since DMF and its metabolites contain nitrogen and oxygen in molecule, it can thus be assumed in the light of the above information that it will be a nitrogen atom rather than an oxygen atom that will participate in the bond with iron. However, one should not leave out of account a likely influence of oxygen on bonds with cytochrome 5-450. This influence could be manifested, e.g. through shifting of the maximum of the examined compounds towards shorter waves (10). It is worth-while stressing that depending on the chemical structure of typical nitrogen ligands significant shifts in the difference spectra of type II (13) are to be observed.

Certain confirmation of the nature DMF and its metabolites bond with cytochrome P-450 is the effect of these compounds on the reduced complex cytochrome P-450-carbon monoxide. It is assumed that change of absorbance of the complex: cytochrome P-450 (Fe^{++})-Co in the presence of the studied compound is an expression of dislodging CO by strong ligands, and it is characteristic of the compounds producing difference spectra of type II (13, 14). Though a visible effect of dislodging carbon monoxide has been observed at relatively high concentrations of DMF, MF and F, however, it proves the opinion that the studied compounds may form complexes with iron of cytochrome P-450. DMF form the strongest complex with cytochrome P-450. This effect can be connected with the presence of two methyl groups in dimethylformamide molecule which may considerably increase its lipophilous features and, thereby, its solubility in lipoproteid microsome membrane and accessibility to the very cytochrome P-450.

In the light of the obtained results it should be assumed that DMF and its metabolites form, with cytochrome P-450 the difference spectra of type II. The nature of the spectrum suggests that the investigated compounds probably bind themselves through nitrogen atom with cytochrome P-450 iron.

In accordance with the reference data (13, 14) the compounds of type II are not, practically, the substrates for the microsomal mixed--function oxidase system, since with oxygen they compete for the bond with iron, and they considerably slow down the reduction of the very cytochrome through the system: NADPH/NADPH-cytochrome P-450 reductase. However, since some substances of this type, e.g. aniline can be hydroxylated with cytochrome P-450 share, the participation of this enzyme in oxidation demethylation DMF requires further confirmation in the studies on microsomal fraction.

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STRESZCZENIE

Dwumetyloformamid i jego metabolity: metyloformamid i formamid tworzą z cytochromem P-450 widmo różnicowe typu II o maksimum w przedziale 417—418 nm i minimum w zakresie 385—392 nm. Związki te prawdopodobnie łączą się z żelazem hemu cytochromu P-450 przez silny ligand azotowy. Największe powinowactwo do tego enzymu wykazuje dwumetyloformamid. Nie wyklucza się również wpływu tlenu na modyfikację widma różnicowego typu II badanych ksenobiotyków.

РЕЗЮМЕ

Диметилоформамид и его метаболиты: метилоформамид и формамид образуют с цитохромом Р-450 дифференциальный спектр II типа с максимумом в интервале 417—418 нм. и минимумом в пределе 385—392 нм. Эти соединения вероятно связываются с железом гема цитофрома Р-450 через сильный азотный лигант. Самое большое сходство с этим ферментом проявляет диметилформамид. Не исключается также влияния кислорода на модификацию дифференциального спектра II типа исследованных ксенобиотиков.