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CYTOCHROME P-450 DIFFERENCE SPECTRA

Effect of Chemical Structure on Type II Spectra in Mouse Hepatic Microsomes

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ABSTRACT

A comprehensive study of the relationship between chemical structure and binding was made with mouse hepatic microsomes. The generally reported type II spectrum (peak 424–435 nm, trough 390–410 nm) is correlated with the presence of a nitrogen atom in which sp^2 or sp^3 nonbonded electrons are "sterically accessible." Structural series showed that as substituents are placed closer to this nitrogen, spectral size is either reduced or eliminated. Other nucleophilic atoms with relatively free steric access may also cause a modified type II binding, *i.e.*, a bathochromic shift. Data are also presented to show that minor structural changes affect both size and type of spectrum.

Cytochrome P-450 has been studied extensively since it was first described (1, 2), with interest centering on its function in the oxidation of endogenous and xenobiotic substrates in many phyla (3–6). In mammals, cytochrome P-450 is found in various organs such as the adrenal cortex (7), liver (2), and other tissues (8), although the hepatic form has been most extensively studied (9). Several interesting spectral phenomena have been noted which distinguish this cytochrome from other *b*-type cytochromes. The first is its binding in the reduced state to carbon monoxide which causes an intense Soret maximum in difference spectrum, at or about 450 nm, from which it derives its name (10). The other distinguishing characteristic is the occurrence of at least two unusual perturbations which may be viewed by optical difference spectroscopy when the oxidized cytochrome is treated with different potential ligands (11).

The first of these perturbations shows a maximum at 385–390 nm and a minimum at 415–420 nm and has been termed "type I" (11), whereas the other, termed "type II," shows a maximum at 424–435 nm and a minimum at 390–410, the variations depending on the source of the enzyme and the nature of the substrate (11). A "type III" spectrum which shows double Soret peaks between 425 and 460 nm has been reported but it involves interaction with reduced cytochrome P-450 (12, 13) and will not be considered here.

Recent work has shown that type I binding may be caused by many compounds, including drugs (11, 14), pesticides (15–17), and steroid hormones (18), from many chemical classes. T'sai *et al.* (19) have stated that type II binding is caused by aromatic amines, although it has also been reported that aliphatic amines (20), isocyanides (12), certain steroids (18, 21), and alcohols (22) cause type II binding.

Although Schenkman *et al.* (14) suggested that the type II interaction represents the formation of a ferrihemochrome from the interaction of a basic amine and a ferrihemoprotein, there are numerous reports (14, 17, 18, 21, 22) of type II binding phenomena with other compounds.

Several recent reports (16, 23–25) explore this problem and in general support the contention (14) that steric and basic features of nitrogen are of primary importance in type II binding. The present investigations define the steric and electronic features influencing type II binding and in addi-

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tion, offer a general method of predicting whether a compound will possess type II character. The companion work expands this to a comparative study of organisms from another phylum.

Materials and Methods

Male mice (5–7 weeks) of the North Carolina Department of Health strain[1] (inbred since 1910) were maintained on Purina lab chow and water *ad libitum*. The mice were killed by decapitation and the excised livers rinsed in cold 0.05 M Tris-1.15% KCl buffer, pH 7.4 (Tris-KCl). After dicing and three additional washes in cold Tris-KCl, the livers were homogenized in an all-glass homogenizer with 4–5 ml of Tris-KCl per liver and centrifuged at 10,000*g* for 15 min. The supernatant was filtered through glass wool to remove the floating lipid layer and recentrifuged at 100,000*g* for 1 hr. All operations were carried out at 4°C. The microsomal pellet was resuspended in minimal amounts of 0.25 M sucrose and either stored at −20°C under nitrogen at a protein concentration of approximately 20 mg/ml or used immediately. Under these circumstances, storage for periods up to 3 months did not influence either type I or type II spectra, although metabolic activity did decrease significantly. All samples were diluted for use with 0.5 M potassium phosphate buffer, pH 7.4, to a final concentration of 1–2 mg/ml.

Spectra were determined between 500 and 350 nm with a Beckman Acta V spectrophotometer equipped with a turbid sample accessory. Zero-difference lines were established before addition of substrate to the microsomes. Solid samples were added directly to the sample cuvette, and both sample and reference cuvettes were inverted 25 times and then permitted to equilibrate in the sample chamber for 5 min. Liquid samples were added to the sample cuvette by microsyringe and an equivalent volume of buffer was added to the reference cuvette before the above procedure was followed. These procedures were repeated until no further increase in spectral size occurred. Anomalous spectra were identified by the use of the criteria outlined below. Spectra are noted as: type I (peak 380–390 nm, trough 415–420 nm), type II (peak 421–435 nm, trough 390–410 nm), type II-A (peak 415–420 nm, trough 380–390 nm), type II-B (peak 435–445 nm, trough 410 nm), or ND (not detectable). Spectral size is defined as the ratio of the peak – trough absorbance difference (ΔA_{max}) to $\Delta A_{450-490}$ for the spectrum of reduced cytochrome P-450-CO (10). The comparison is made on the basis of absorbance units and does not involve extinction coefficients. This was found to be repeatable within a range of ±20% with microsomal preparations from individual mice killed on different days, or to ±10% with livers of 20 or more mice which were pooled before homogenization. All values are the average of at least two determinations, or more in key instances.

In an attempt to further eliminate variations, structural series of compounds were run on the same day with the same microsomal preparations. Although the variation cited above occurred, it invariably affected all compounds in a series in the same manner thus providing an additional check on procedures.

Chemicals were obtained commercially except where noted[2] and the purity was checked by gas or thin-layer chromatography. Materials were purified as needed by conventional techniques.

To minimize misinterpretation of data, we have attempted to establish formal guidelines which will indicate when spectral perturbations have been caused by nonlinear baseline changes due to a turbidity difference between sample and reference (26); mixed interactions, such as the simultaneous formation of type I and II spectra;[3] denaturation of cytochrome P-450 (to P-420 or other species;[4] native absorbance of added ligand(s). The guidelines are that: the isosbestic point must remain constant as ligand concentration is changed; increasing concentrations of a ligand must show concomitant increases in maxima and decreases in minima; no noticeable absorbance inflection may be apparent between 500 and 470 nm; the absorbance change at 360 nm must be less than 50% of ΔA at the nearest maximum or minimum and be positive for type I and negative for type II.

Results and Discussion

Preliminary work with a random selection of pesticides of varying chemical structures, including organophosphates, carbamates, chlorinated hydrocarbons, and botanicals, suggested that classical type II binding might be related to the presence of an "accessible" nitrogen atom but not any other chemical grouping (16), in agreement with Schenkman *et al.* (14). Pyridine is a well known type II compound and inasmuch as the aromaticity of the molecule gives it a planar structure that could simplify structure-activity correlations, the cytochrome P-450 difference spectra of a series of substituted pyridines were examined. The results

[1] It has become apparent since the completion of this work, that the mouse strain used may have been contaminated with the DUB-ICR strain (Flow Research, Duplin, Va.). No significant differences have been seen between the two strains with respect to the findings described in this communication.

[2] The following compounds were generously donated: 6-methylbenzothiadiazole, 5-nitrobenzothiadiazole, and 4-chlorobenzothiadiazole by C. F. Wilkinson, Cornell University; 1-(1-naphthyl)imidazole, 1-(2-cyanophenyl)imidazole, and 1-(2-isopropylphenyl)imidazole by E. Stevenson, E. I. DuPont de Nemours and Co. (Wilmington, Del.).

[3] R. Mailman, A. P. Kulkarni, J. P. Service, and E. Hodgson, in preparation.

[4] R. B. Mailman and E. Hodgson, in preparation.

obtained with methyl substituents (table 1) clearly illustrate that substitutions on carbons 2 and 6 tend to inhibit or abolish spectral binding, whereas substitutions at carbons 3(5) or 4 have no such effect. The same pattern is seen with carboxyl substitutions, although the size of all spectra are reduced, due presumably to decreased lipophilicity. The substituted N-oxides of pyridine follow a similar pattern of substituent effect, although the unsubstituted N-oxide does not, presumably due to a significant difference in lipophilicity. The interaction of these compounds is discussed more fully later.

Nonaromatic cyclic amines were next examined since they also possess a degree of steric restraint. The pattern, relative to steric hindrance, observed (table 2) with these amines was consistent with that obtained with the substituted pyridines. In this case, pyrrolidine gives a type II spectrum, but substitution of large alkyl groups on the nitrogen eliminates type II binding and type I binding is observed instead. This same effect is seen when a cyclohexyl moiety is added to the nitrogen of 2-pyrrolidone. The series of piperidine derivatives gave results which, although complex, were consistent with the above hypothesis. These include a mixed type II and type I spectrum[3] similar to that reported by Schenkman (27), as well as a new concentration-dependent spectrum, previously unreported.[4]

TABLE 1

Effect of molecular substitutions on cytochrome P-450 difference spectra of pyridine analogs with mouse hepatic microsomes

Substituent	Spectral Type	Spectral Size
—	II	0.540
4-Methyl	II	0.620
3-Methyl	II	0.654
2-Methyl	II	0.380
2,6-Dimethyl	II	0.010
4-Carboxyl	II	0.246
3-Carboxyl	II	0.140
2,4-Dicarboxyl	II	0.009
2-(2-Propane-1,3-diol)	II-A	0.077
3-Acetyl	II	0.407
3-Pyrollidino	II	0.264
3-(N-Methylpyrollidino)	II	0.162
4-(4-Nitrobenzyl)	II	0.516
4-(Methyl phenyl urea)	II	0.550
N-Oxide	II	0.221
N-Oxide, 2-methyl	II	0.250
N-Oxide, 3-methyl	II	0.496
N-Oxide, 4-methyl	II	0.496

TABLE 2

Mouse hepatic cytochrome P-450 optical difference spectra of cyclic amines

Compounds	Spectral Type	Spectral Size
5-Member ring		
Pyrrolidine	II	—[a]
1-Cyclohexenylpyrrolidine	I	0.165
1-Cyclopentenylpyrrolidine	I	0.156
2-Pyrrolidone	II	0.088
1-Cyclohexyl-2-pyrrolidone	I	0.106
3-Pyrroline	II	0.059
Pyrrole	II	—[a]
6-Member ring		
Piperidine	II	—[a]
3-(1-Piperidino)propanol	II	—[a]
4,4-Dipiperidine	II	0.210
N-Ethylpiperidine	II	—[a]
2-Methylpiperidine	II	0.146
2-Ethylpiperidine	I and II	—[b]

[a] These compounds gave an unusual and previously unreported type II spectrum which showed a characteristic concentration-dependent change in position of maxima and minima. Since this complicates the determination of peak-trough differences, spectral type only is reported. A more detailed analysis of this spectral type is in progress and will be reported later.[4]

[b] Spectrum appears to be a mixture of types I and II.[3]

A comparison of the binding of indole and indoline (table 3) shows distinctly that subtle changes may have a significant effect on observed spectra. Indoline gives a strong type II spectrum, whereas indole gives a type I spectrum. The difference between these molecules is the unsaturation at carbons 3–4, causing a "stiffening" of indole. This causes a change in accessibility to the nonbonded nitrogen electrons. It also appears that conformation changes affect the differences in binding of pyrrole and 3-pyrroline (table 2).

The report that many carbamate insecticides do not form type II spectra despite the presence of nitrogen (16) led to the examination of a series of related compounds. The N-methyl amides (table 3) showed that the smaller substitutions gave type II spectra, and as the size of the acyl moiety increased above that of the acetamide, binding was diminished. The other compounds in table 3 gave results consistent with the hypothesis presented earlier.

TABLE 3

Mouse hepatic cytochrome P-450 optical difference spectra of heterocyclic compounds and amides

Compounds	Spectral Type	Spectral Size
Heterocyclic		
6-Methylbenzothiadiazole	II	0.080
5-Nitrobenzothiadiazole	ND[a]	—
4-Chlorobenzothiadiazole	ND	—
Indole	I	0.074
Indoline	II	0.383
Benzimidazole	II	0.500
1-(1-Naphthyl)imidazole	II	0.595
1-(2-Cyanophenyl)imidazole	II	0.592
1-(2-Isopropylphenyl)imidazole	II	0.630
3-Aminotriazole	II	0.170
Amides		
N-Methylformamide	II	0.094
N-Methylacetamide	II	0.265
N-Methylpropionamide	II	0.068
N-Methylbenzamide	ND	—

[a] ND, not detectable.

TABLE 4

Mouse hepatic cytochrome P-450 optical difference spectra of amines

Amines	Spectral Type	Spectral Size
Methylamine HCl	II	0.274
Dimethylamine HCl	II	0.040
Ethylamine HCl	II	0.197
Diethylamine HCl	I	0.064
Triethylamine	I and II	—[a]
n-Propylamine	II	0.234
n-Butylamine	II	0.386
Octylamine	II	0.910
Benzylamine	II	0.828
Aniline	II	0.452
4-Chloroaniline HCl	II	0.288
4-Anisidine (4-methoxyaniline)	II	0.297
4-Chloro-N-methylaniline HCl	II	0.053
1-Naphthylamine	I and II	—[a]

[a] See footnote [b], table 2.

TABLE 5

Mouse hepatic cytochrome P-450 optical difference spectra of CN-containing compounds

Compound	Spectral Type	Spectral Size
Alkylnitriles		
Acetonitrile	II	0.037
Propionitrile	I and II	—[a]
3-Methoxypropionitrile	I	0.013
2,2-Azobis(2-methylpropionitrile)	I	0.032
NaCN	II-B	1.24
Benzonitrile substitution		
—	I and II	—[a]
2-Chloro	II	0.025
3-Chloro	I	0.107
4-Chloro	II	0.042
3,5-Dichloro	ND[b]	—
2,6-Dichloro	I	0.064
3,5-Diiodo-4-hydroxy	II	0.275
4-Hydroxy	II	0.350
3-Hydroxy	II	0.230
2-Hydroxy	II	0.281
4-(Dimethylamino)	I and II	—[a]
4-Methoxy	I and II	—[a]

[a] See footnote [b], table 2.
[b] ND, not detectable.

Various series of amines were examined (table 4). The monoalkylamines gave distinct type II spectra as expected with spectral size increasing when chain length became greater than 3. However, the di- and trialkylamines clearly indicate that as steric access to the nitrogen is hindered, type II binding is abolished or hindered (25). The series of aniline compounds showed that electronic effects of substituents may have an effect (aniline *vs.* 4-chloroaniline), either by inductive effects on the nitrogen or by altering lipophilicity, whereas the previously noted effect of steric hindrance is still apparent (4-chloroaniline *vs.* 4-chloro-N-methylaniline).

The situation with the nitriles is more complex (table 5). Although steric accessibility is not a factor, the alkylnitriles gave very small or no type II spectra. This is consistent with the fact that these compounds have nonbonded electrons with primarily *sp* character and are therefore less likely to cause bathochromic shifts. Benzonitrile gave a small mixed spectrum[3], whereas 2- or 4-substitutions with chlorine or dimethylamine moieties increased the type II interactions. Conversely, the 3-chloro or 3,5-dichloro compounds did not give type II binding. (The 2,6-dichloro substitution is presumably sterically hindered and thus the type I spectrum obtained is not unexpected.) The complexity increases when considering the hydroxybenzonitriles. The 2,3, and 4-cyanophenols (hydroxybenzonitriles) all gave strong type II spectra. Although the 2- and 4-substituted compounds gave larger spectral sizes than the 3-substitution, as would be expected from a 2,4-electronic effect, the

3-substitution did give a strong unexpected type II spectrum[5]. It appears, however, that because 3,5-diiodo-4-hydroxybenzonitrile also gave a strong type II spectrum, the occurrence of type II binding is caused by the nitrile group rather than the hydroxyl moiety because when the hydroxyl group is hindered, as in 3,5-diiodo-4-hydroxybenzonitrile, little effect is seen on spectral size.

This is borne out by the results with a series of substituted phenols (table 6). The 2- and 4-amino group gave larger spectral size than the 3-amino group. However, 4-methoxyphenol did not give a spectrum of detectable size while the 4-methylmercapto group caused a mixed spectrum[3]. Phenol in low concentrations ($<10^{-3}$ M) gave a very small type II spectrum with a peak at 425 nm and a trough at 410 nm. Additional amounts of phenol caused an increase in spectral size and a slight change in λ_{max}. This was, however, due to denaturation of P-450, the appearance of cytochrome P-420 being readily apparent in the CO spectrum.[3]

The results with the thiocyanates and phenyl isothiocyanate illustrate again the importance of the electronic state of the nitrogen to the ability of a compound to cause a type II spectral shift. Only the salt, potassium thiocyanate, gives a type II spectrum, consistent with the difference between certain charged species and their organic derivatives. A comparison between NaCN and the four alkylnitriles listed in table 5 is a further illustration of this point.

The data in table 7 raise an interesting question which has been discussed at length elsewhere (22, 29, 30), that is, the cause of the modified type II (inverse type I) spectrum, which we call type II-A (22, 27, 31). The simplest interpretation of our data is that oxygen atoms act much like nitrogen atoms, that is, as nucleophiles replacing another ligand at the 5th or 6th ligand position of the heme group of P-450, causing a bathochromic shift. It is of lower intensity and shorter wavelength due to the lower nucleophicity of the groups involved. As with the amines, as chain length increases binding

TABLE 6

Mouse hepatic cytochrome P-450 difference spectra of substituted phenols and miscellaneous nitrogen compounds

Compounds	Spectral Type	Spectral Size
Phenols		
—	II	—[a]
4-Methoxy	ND[b]	—
3-Methyl	I	0.093
4-Methyl	I	0.155
2-Amino	II	0.280
3-Amino	II	0.160
4-Amino	II	0.308
4-(Methylmercapto)	I and II	—[c]
Thiocyanates and isothiocyanates		
Potassium thiocyanate	II	0.290
4-Nitrobenzyl thiocyanate	I	0.160
Phenyl isothiocyanate	I	0.230
Thanite (isobornyl thiocyanatoacetate)	I	0.240
Isonitrile		
Ethyl isocyanide	II-B	1.17

[a] See footnote [a], table 2.
[b] ND, not detectable.
[c] See footnote [b], table 2.

[5] These particular compounds were twice recrystallized and final products had sharp melting points identical with those cited in ref. 28.

TABLE 7

Mouse hepatic cytochrome P-450 difference spectra of alcohols and other oxygen-containing compounds

Compounds	Spectral Type	Spectral Size
Alcohols		
Methyl	II-A	0.049
Ethyl	II-A	0.111
n-Propyl	II-A	0.121
n-Butyl	II-A	0.141
n-Hexyl	I and II-A	—[a]
tert-Amyl	I	0.010
sec-Butyl	I	0.083
Isoamyl	I and II-A	—[a]
Glycerol	ND[b]	—
Nonaromatic oxygen-containing		
Acetaldehyde	I	0.090
tert-Butylmethyl ketone	I	0.210
3-Methyl-3-pentene-2-one	II-A	0.072
Glycolacetate	II-A	0.021
Ethanolamine	II	0.081
Tetrahydrofuran	ND	—
Steroids		
Progesterone	ND	—
Hydrocortisone 21-sodium succinide	II-A	0.053
Estrone	ND	—
β-Estradiol	ND	—

[a] See footnote [b], table 2.
[b] ND, not detectable.

increases due to greater lipophilicity. When the access to the oxygen is hindered or the lipophilicity is too great either mixed spectra (type II-A and type I) or type I binding occurs, also as with the amines. In addition, type II-A spectra frequently have a different λ_{max} from that which would be obtained by reversal of a type I spectrum. Further, spectra which may be mixtures of types I and II-A are obtained which would also seem to argue against "endogenous substrate displacement" (22, 31) as the cause of the interaction. In addition, the carbanion-like species of the isonitriles gives what we term a II-B difference spectra (still longer wavelength), possibly because of greater nucleophilicity of this species. Another metabolite-P-450 complex which may result from a carbanion-like species also produces a type II-B spectrum (13, 29).

Although this interpretation is open to question, the remaining data in table 7 suggest that the criteria established for the nitrogen of nitrogen-containing compounds may also apply to other nucleophilic atoms. For example, molecules containing sterically accessible oxygen atoms give type II-A spectra in many cases. This of course does not eliminate the possibility of an inverse type I spectrum being caused by displacement of endogenous substrate but it does suggest this is not always the case. The remaining data (table 8) fit the steric accessibility hypothesis. In the zwitterionic state, the nitrogen atoms of amino acids have no nonbonded electrons, and therefore show no type II spectra. Alternately the molecule may be too polar to penetrate the hemoproteins. Histidine gave a type II spectrum due to its imidazole moiety, but much weaker than substituted imidazoles (table 3).

TABLE 8

Mouse hepatic cytochrome P-450 difference spectra of amino acids and miscellaneous compounds

Substituent	Spectral Type	Spectral Size
Amino acids		
L-Histidine	II	0.067
L-Arginine HCl	ND[a]	—
L-Tryptophan	ND	—
L-Methionine	I	0.015
L-Glutamic acid	ND	—
L-Lysine	ND	—
L-(N-acetyl)Methionine	I	0.040
Miscellaneous		
3-Quinuclidinol	I	0.145
Hexobarbital	I	0.040
Phenobarbital	ND	—
2-Thiobarbituric acid	II	0.072
Cyclophosphamide	ND	—
SKF 525-A	I	0.200
Phenacetin	II	0.155
Piperonyl butoxide	I	0.220
Benzphetamine	I	0.210

[a] ND, not detectable.

In summary, we have further delineated the phenomenon of type II spectral bindings with hepatic microsomes. Nitrogen-containing compounds in which the nitrogen is primarily an sp^2 or sp^3 hybrid and in which the nonbonded electron pair is accessible will cause type II spectra. Electronic effects of 2- or 4-substituents can also affect spectral size by changing these parameters. However, substituents with different electronic effects (*e.g.*, 4-chloro- and 4-methoxyaniline; benzonitriles) may cause similar spectral effects. Because changes, by induction or resonance, in the electronic configuration of the nitrogen atom, as well as the polarity of the molecule, will influence spectral perturbations if the stereochemical effects are not significant, these findings require further examination. We believe, however, that other nucleophilic atoms, similar with respect to accessibility and electronic state, can also cause modified type II binding. This presents an alternate explanation of some cases of modified type II binding frequently regarded as "inverse type I." These modified type II interactions occur at characteristic wavelength ranges that may overlap that of nitrogen (424–435 nm) type II spectra.

The amines provide a useful series for observing the effect of dissociation on spectral size and type. The monoalkylamines have similar pK_a values (ethyl 10.81, butyl 10.77, propyl 10.71, methyl 10.66, octyl 10.65), whereas the spectral sizes ranges from 0.197 to 0.910 in the following order: octyl > butyl > methyl > propyl > ethyl. The three amines listed in table 4 which gave type I spectra had pK_a values of 3.92 (naphthylamine), 10.49 (diethylamine), and 11.01 (triethylamine), whereas the 11 that gave type II spectra had pK_a values which varied from 4.15 (chloroaniline) to 10.81 (ethylamine).

Some significant differences exist between the results from different laboratories. N-oxides of pyridine give us strong type II interaction whereas Temple (30) reported them to be type I com-

pounds. This may be due to compound impurities. Uehleke (32) reports intense anomalous spectra (maximum 450 nm, minimum *ca.* 410, $\Delta A >$ reduced CO-complex) with nitrosobenzene and phenyl hydroxylamine, whereas Temple found they were type I substrates (30). Most reports, although fragmentary, agree with the findings presented (14, 33-35). Schenkman *et al.* (14) reported that a type I spectrum was obtained with phenobarbital and rat liver microsomes. Extensive examination, under a variety of experimental conditions, failed to reveal any discernible difference spectrum when phenobarbital was added to mouse liver microsomes. Whether these represent strain, species, or procedural differences is not presently clear. However, since induction of hepatic enzymes can cause qualitative as well as quantitative changes in cytochrome P-450 (36-38), difference in spectral behavior should be expected with different cytochromes.

We have not attempted to deal with the problem of modifiers, symmetrical-asymmetrical spectra, or the effect of adding more than one ligand to the same preparation (39, 40). Saturating levels of all ligands were used since we were attempting to define the factors causing type II spectra (bathochromic shifts), and at lower concentrations many type II ligands will give type I spectra (41). Since even type I compounds may not be metabolized (15, 42, 43), it is inappropriate to discuss the metabolic implications of difference spectra. The major legitimate use of types I and II difference spectra should be for characterization and comparison of different cytochromes. If, ultimately, this approach yields a general chemical description of the cytochrome molecule in the vicinity of the porphyrin moiety, metabolic implications may then be apparent.

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