

UNIT II: Liver, Metabolism and Chemical Carcinogenesis

Assigned Reading:

Research Landmarks: "The presence and significance of bound aminoazo dyes in the livers of rats fed p-dimethylaminoazobenzene" In: The Journal of NIH Research 2:66-78 (1992).

Assignment #2: Pick any xenobiotic metabolizing enzyme and provide information that tells of a) the type of reaction it catalyzes, b) its role in carcinogen activation or detoxification (if known), c) its substrate specificity. Most importantly, try to provide two examples of substrates which demonstrate broad substrate "specificity"

Historical Background

In the early 20th century, the etiology of cancer was thought to be either; 1) viral, 2) chemical or 3) genetic. Although each school of thought often predicted that "their mechanism" would be universal, each of these ideas has been proven to be correct. We now know that each factor can be important to human cancer incidence. The story of a chemical etiology for cancer began with work from the 18th and 19th century Europe where physicians had noted correlations between snuff use and mouth cancer and a high incidence of scrotal cancer in chimney sweeps. The story of the English physician Percival Pott is often recounted as a primary event in our understanding of chemical carcinogenesis. In addition, his observation led to an early scientific focus on the mechanism of action of "coal tar" carcinogens. The early coal tar connection led to the first chemical carcinogenesis experiment by Yamagiwa and Ichikawa who showed that rabbit ears painted with coal tar developed skin tumors. This observation led to the isolation and structural elucidation of the first carcinogens, dibenzanthracene and benzo(a)pyrene by Kennaway and Cook between the 1930s and 1950s (for a brief review of the history of cancer research see J. NIH Research 4:92-94).

Genotoxic Carcinogens

Looking for a single explanation: Experimental and epidemiological observations made in the middle of the 20th century had identified a number of chemicals that could cause cancer in humans or experimental animals. Coal tar carcinogens like benzo(a)pyrene, pesticides like AAF, and azo dyes like DAB were among the first generation of chemical carcinogens to be studied. An important problem in these early days was to identify the structural characteristics common to all chemical carcinogens. This was an especially confusing problem, since they appeared to be no clear structural similarities between the known carcinogens. For example look at these proven liver carcinogens. **What structural features do they have in common that could predict carcinogenicity?**

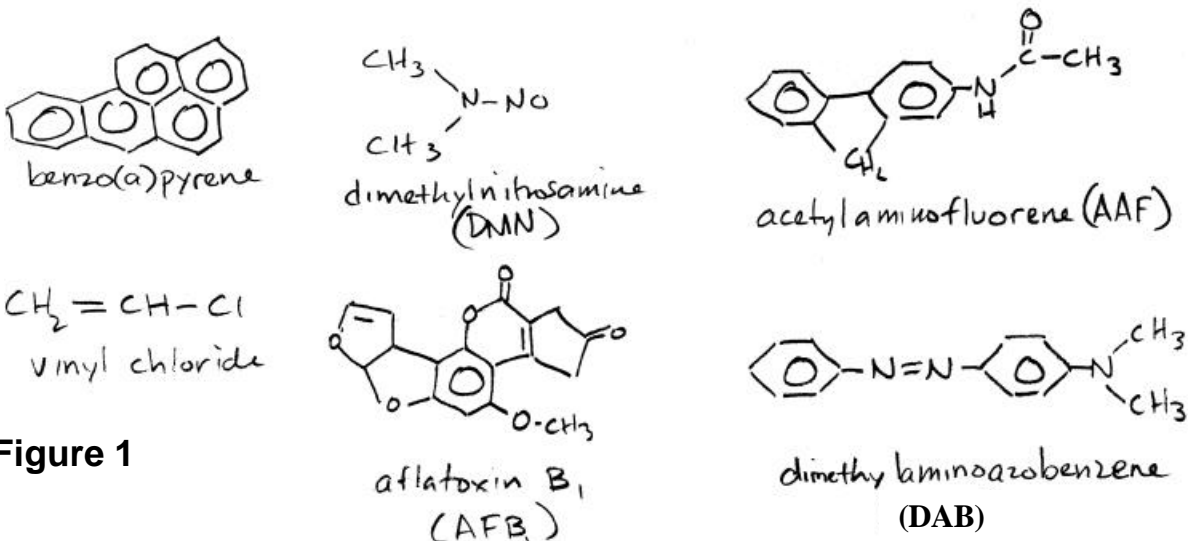


Figure 1

Covalent modification of DNA by chemical carcinogens: An experiment that changed the way we think about chemical carcinogenesis was performed here at the McArdle Laboratory in 1947 (assigned reading). During this time, the ideas were that a carcinogenic chemical might activate a virus or alter "heritable entities". The Millers were working on the carcinogenic mechanism of DAB (see structure above). This compound was a member of the azo dye family. They were trying to understand the metabolism of this compound when they

stumbled upon an interesting observation: The livers of the treated rats were dyed pink. More importantly this dye was covalently bound to cellular protein (TCA precipitate), primarily in the liver where DAB caused cancer. When these dyes were added to liver protein in vitro, they were readily removed by organic solvents (EtOH or acetone). Coupled with the above observations, these data suggested that the dyes covalently bound to protein and this event required an in vivo step, presumably metabolism. **Although this paper represents a significant advance, what is the error that is obvious in hindsight? What are the three experimental components of the proof that DAB is carcinogenic via metabolism/covalent modification of macromolecules?**

TABLE II: THE LEVEL OF TOTAL BOUND DYE IN THE TISSUES OF RATS FED p-DIMETHYLAMINOAZOBENZENE (Average of 2 rats; diets fed for 2 months)

Tissue*	E/100 mgm. powder			Color of acid-ethanol solution	
	Basal diet + dye	Basal diet	Bound dye†	Basal diet + dye	Basal diet
Liver	0.330	0.050	0.280	Pink	Light yellow
Lung	0.044	0.030	—	Light yellow	" "
Heart	0.088	0.058	—	" "	" "
Kidney	0.074	0.054	—	" "	" "
Skeletal muscle	0.038	0.038	—	" "	" "
Small intestine	0.034	0.046	—	" "	" "
Blood plasma	0.078	0.018	0.060	Light pink	Nearly colorless
Red blood cells	0.356	0.332	—	Red-brown	Red-brown
Spleen	0.304	0.190	—	" "	" "

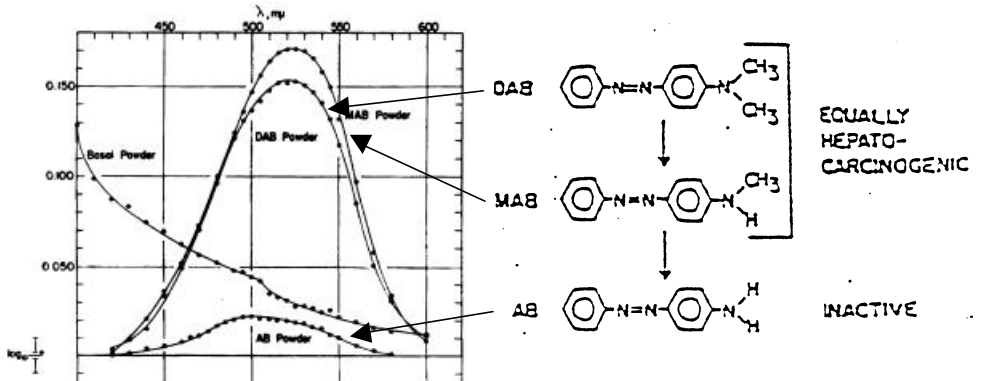
TABLE III: THE LEVELS OF FREE AND BOUND AMINOAZO DYES IN THE LIVERS AND BLOOD OF ANIMALS FED p-DIMETHYLAMINOAZOBENZENE (Averages of 2 to 5 animals diets fed for 2 months)

	Free aminoazo dyes					Bound aminoazo dye E/100 mgm. liver powder	Susceptibility to induction of liver tumors with DAB
	Liver (perfused) µgm./gm. fresh wt.			Blood µgm./ml.			
Albino rat (Sprague-Dawley)	DAB*	MAB*	AB*	DAB	AB	0.236	High (5,24)
Albino mouse (abc)	0.32	0.19	1.83	Not detectable	13.4	0.070	Low (5,6,24)
Guinea pig†	0.17	0.11	2.20	0.46	83.0	Not detectable‡	None (21)
Rabbit†	Not detectable			Not detectable			Very low (5)
Cotton rat†	0.09	0.01	1.54	Not detectable	1.55		Very low (5)
Chicken (white leghorn)	0.04	0.04	0.84	"	11.9		(Present authors) None (5)

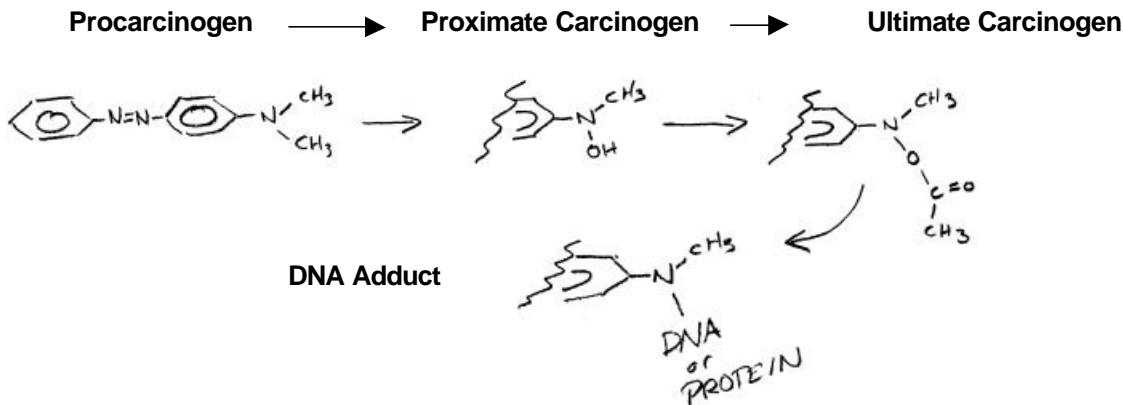
*DAB=p-dimethylaminoazobenzene. MAB=p-monomethylaminoazobenzene. AB=p-aminoazobenzene
 †Strain unknown
 ‡The final acid-ethanol solutions were light yellow and gave uncorrected E values of 0.062-0.068 (normal rat liver = 0.050).

*The blood was removed by perfusion of the whole rats.
 †Only in the case of liver and blood plasma do the differences in absorption and color of the extracts from tissues of normal and dye-fed rats indicate the presence of bound dye. In the case of the other organs the small differences can probably be ascribed to differences in the amount of residual blood and other pigments. The spleens of rats fed the azo dye were engorged with red cells and their degradation products which were not removed by perfusion.

Data from the Miller's 1947 paper. Note that "E" indicates the absorption of the dye bound to precipitated protein as determined by spectroscopy. Table III: The numbers in parentheses are references that can be found in the original paper.

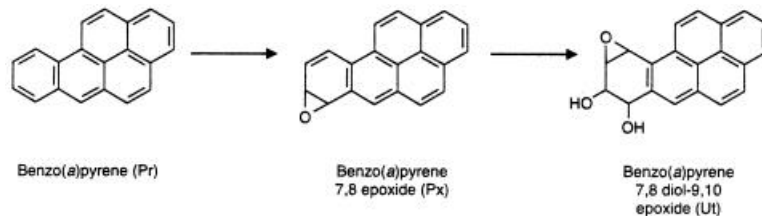


One of the most important conclusions from the above experiments (and those that soon followed) was that the activity of carcinogens was related to their ability to covalently modify DNA, "the heritable material". Furthermore, for many carcinogens, this process required metabolism. In addition to the idea of covalent modification, these experiments demonstrated the importance of carcinogen metabolism and suggested the following scheme (and nomenclature). Procarcinogens are the parent compounds that cause cancer yet are metabolically upstream from the damaging agent. Ultimate carcinogen refers to the reactive metabolite that covalently modifies DNA. Proximate carcinogen is an intermediate species between pro and ultimate (a metabolic step along the way). The actual metabolic bioactivation pathway for DAB is shown below.

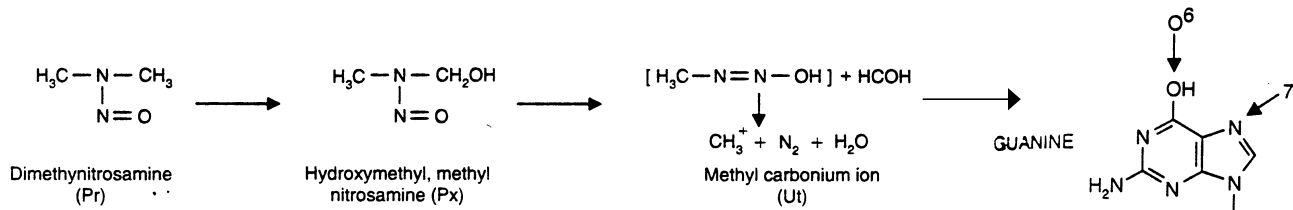


Two more examples:

1) Benzo(a)pyrene: Experiments with benzo(a)pyrene provide an additional example of the types of experiments that led to the development of the electrophilic theory of chemical carcinogenesis. Benzo(a)pyrene is the coal tar carcinogen isolated by Kennaway. It is also found at high levels in charcoal broiled foods, cigarette smoke and in diesel exhaust. Its pathway was worked out by a number of laboratories and the experiments can be reviewed in Cancer Research 42:4875. The experiments were initiated by the observation that radiolabeled benzo(a)pyrene was found bound to DNA after being painted on skin. This led to attempts to identify the metabolic steps, as well as the proximate and ultimate carcinogens. The basic assay system used was the ability of a chemical to covalently modify DNA in solution (in vitro). **Presume that the following scheme is the route to DNA damage and the ultimate carcinogen (see below). Using only purified DNA, purified and/or radiolabeled compounds of any structure you need and tissue homogenates, how would you 1) provide proof for the metabolic path (identify pro, proximate and ultimate carcinogen), 2) prove that metabolism is required and 3) identify the DNA base or proteins that have been modified? You don't need to know much chemistry to answer these questions.**



2) Nitrosamines: Nitrosamines were first discovered in a study of workers who were exhibiting jaundice and liver damage as a result of occupational exposure to an unknown agent. Nitrosamines have been used in a number of industries including the production of rubber. In the ensuing animal experiments, dimethylnitrosamine (DMN) was found to be extremely hepatotoxic to rats and caused a high incidence of liver tumors. In fact, it is one of the most carcinogenic compounds known. A metabolic bioactivation pathway to a highly reactive "ultimate carcinogen" that can add a methyl group to the O6 and N7 of guanine was determined.



The electrophile theory of chemical carcinogenesis. Based upon results like those described above, a mechanism was proposed that explained what all of the carcinogens in Figure 1 had in common. 1) They were all metabolized to a compound that could covalently modify DNA, the genetic material. 2) The ultimate carcinogen was typically an electrophile. An electrophile is a highly reactive ("electron loving") compound that will covalently interact with nucleophilic centers. In biological systems, nucleophilic centers are atoms with unpaired electrons, e.g., O⁻, N⁻, S⁻. Proteins, RNA, DNA and all biological molecules are loaded with nucleophilic centers. The metabolites of the carcinogens shown in figure 1 modify DNA by direct covalent attachment. Therefore, they are often called alkylating agents. **Can you think of another class of alkylating agents that are important in cancer biology?**

Direct acting carcinogens. A variety of carcinogens have been found that does not require metabolic activation. Such compounds are called direct acting carcinogens. An example of this class of compounds is N-methylnitrosourea (NMNU, (CH₃NNHCONH₂)). This compound is highly reactive without metabolism, especially in aqueous systems where they generate the methyl carbonium ion described above (CH₃⁺). Experimentally, direct acting carcinogens like NMNU can be powerful tools. You do not have to worry about metabolism and they are so unstable that they do not last long in the experimental system. When you directly expose animals to metabolic end-products like benzo(a)pyrene diol-epoxide or acetylated form of MAB, you effectively have a direct acting carcinogen. **With this in mind, how do you think site of carcinogenic action will change as you expose animals to procarcinogens, proximate and ultimate carcinogens?**

Carcinogen Metabolism:

The concept of carcinogen metabolism may seem a bit odd. Especially since we are often taught that enzymes have great stereospecificity (the lock and key idea). It turns out that all organisms including humans have a considerable capacity to metabolize (alter covalent bonds) of almost any chemical structure. From an evolutionary perspective, this ability probably arose from a need to adapt to a variety of environmental chemical stresses. The natural chemical environment is not benign, it is loaded with toxic compounds and always has been. Naturally occurring plant alkaloids, mycotoxins, venoms, byproducts of fire etc have been around longer than the human species. If an organism can't metabolize these compounds it will not survive. To combat this problem, a system has evolved that is directed toward making low molecular weight lipophilic molecules more polar and excretable. To simplify, carcinogen metabolism is commonly classified as Phase I and Phase II. This nomenclature arose from the initial thought that Phase I reactions always preceded Phase II. This is now known not always to be the case.

The role of the liver:

Phase I and II metabolic activities are present in all cells, but different cell types have unique constituencies. To a large extent, it is the particular metabolic constituency of a cell that will dictate which electrophiles are generated in that cell. To a large extent, species specificity of a carcinogen can also be determined by the presence or absence of a particular metabolic capability at a given site. Given the liver's place in the front line (downstream of the GI tract and upstream of the general circulation), it is not surprising that the liver has the highest concentration of enzymes with the capacity to metabolize carcinogens and toxicants (LIVER > LUNG > KIDNEY = INT. = ADRENALS > OTHER). Thus, liver metabolism of carcinogens can have a significant impact on all other sites. For many drugs, the liver can remove more than 90% of a drug before it ever reaches the systemic circulation. **Why does it "make sense" that the liver would have the greatest capacity to metabolize carcinogens?**

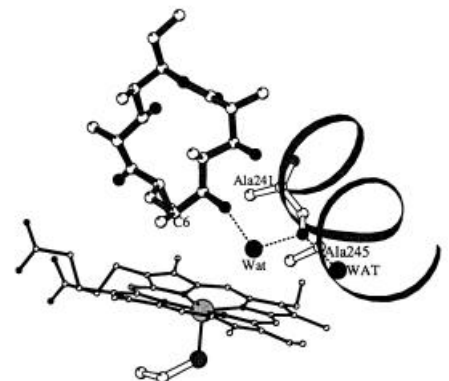
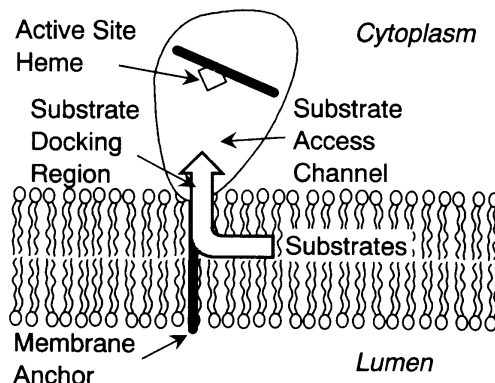
Examples of Phase I metabolic systems.

Phase I enzymes carry out simple metabolic reactions, oxidations, reductions and hydrolysis. Thus, they basically add or subtract protons, add oxygen or add water.

Cytochrome P450.

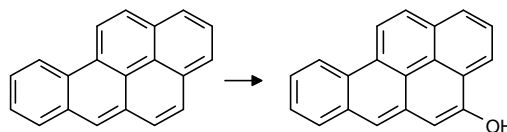
(also called mixed function oxidases or P450s) They are called P450 because they have an iron-heme center at their active site and absorb light at 450 nm. These enzymes are found in the smooth endoplasmic reticulum (ER). Experimentally, P450s are found in the "microsomal fraction of cell homogenates (a 100,000 x g pellet). These genes are members of a large superfamily. The nomenclature is to call them all CYP and then give them a numerical designation based upon their phylogenetic relationship to other members. For example CYP1A1, CYP1A2, CYP2D6 etc. In the human there are 39 known CYP genes and 10 pseudogenes. They map all over the genome and are not usually clustered. They are found in all organisms (prokaryotic and eukaryotic). In the rat there are 60 known CYP genes and in the mouse there are 43. Assuming that we are only about half done finding them, there are likely to be many more. Most if not all of these CYPs have metabolic activity towards foreign chemicals (carcinogens among them). This does not mean that carcinogen metabolism is their only physiological role. Many are involved in metabolism of steroids, eicosanoids and other lipid soluble biological signaling molecules. Due to the flexibility in the ligand-binding pocket, each P450 has the capacity to oxidize a variety of different substrates

Left, a schematic of how a lipid soluble substrate enters the p450 active site. Right, a close up of the heme active site in P450 where oxidation occurs. Taken from Cytochrome P450: Structure Mechanism and Biochemistry (Ortiz de Montellano ed.) 2nd ed. Plenum Press, New York (1995).

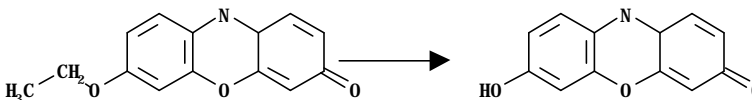


P450 reactions that are of importance to chemical carcinogenesis.

Aromatic oxidation_ Benzo(a)pyrene is hydroxylated to a series of phenols. These same enzymatic mechanisms also yield the epoxides which can be electrophilic (see above).



Oxidative Dealkylations: The oxidation of ethoxyresorufin (a one-time pesticide) leads to an unstable hydroxylated intermediate that spontaneously dealkylates. This reaction is similar to the oxidation that leads to the demethylation of (DMN see above). This particular reaction is shown to demonstrate that flexibility of these enzymes. The enzyme that catalyzes this reaction also catalyzes the above benzo(a)pyrene reaction (CYP1A1).



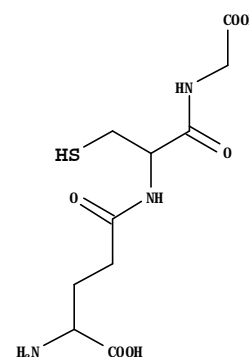
Other P450 reactions: Other important reactions include cleavage of azo bonds and hydroxylation of amines. **How might these two activities influence the carcinogenicity of DAB (see above)?**

NON P450 catalyzed Phase I reactions.

P-450s do not catalyze all Phase I. Reactions. For example monoamine oxidases are located in the outer mitochondrial outer membrane and can act on a wide variety of catecholamine analogues. Alcohol and aldehyde dehydrogenases (soluble oxidases, primarily in the liver). These dehydrogenases can act on a variety of alcohols and aldehydes.

Epoxide hydrolase: One form is cytosolic another is in the ER. Can metabolize the cytotoxic and carcinogenic benzo[a]pyrene epoxides to diols (see above). These diols can either serve as a route to the ultimate electrophile or can be readily excreted. These EH enzymes provide a good example of how the same enzyme can metabolize foreign and endogenous compounds. For example EH can hydrolyze epoxides of benzo(a)pyrene and cholesterol.

Nucleophilic targets in cells. The electrophilic attack on cellular nucleophiles lies at the center of an important mechanism of chemical carcinogenesis. In addition, it explains much of the cytotoxicity of many poisons and carcinogens. High doses of any of the carcinogens listed in table 1 will also lead to cell death and necrosis at a number of sites, especially liver. This toxicity is due to disturbances in protein structure and function, as well as the function of RNA, DNA, carbohydrates and lipids. One important mechanism that cells have to prevent this cell damage is to have high concentrations of cellular nucleophiles floating around. Most important among these is a compound called glutathione (GSH). GSH is a unique tripeptide (gamma-glutamyl-cysteinyl-glycine). Cytosolic concentrations range from 0.5-10 mM. Given the liver's important role in chemical defense, its GSH levels are very high, around 8 mM. The most important element of GSH is the thiol. It provides a highly nucleophilic center (unpaired electrons) that react with a variety of weak and strong electrophiles. One important aspect of GSH is that it can be depleted by continual chemical stress. Thus continued chemical exposure can yield much different effects than short term exposure. In addition, the cells take measures to conserve GSH. One way is to make sure that the entire molecule is not excreted. Much of the GSH is recycled prior to excretion. The final product bound to the electrophile is often called a mercapturate. Finally, GSH plays an important role providing reducing equivalents to protect cells from peroxidative damage. As a result of this, some GSH may be in an oxidized form in the cell, typically as a dimer referred to as GSSG. **For DAB or benzo(a)pyrene, would you expect to get greater DNA/protein binding using microsomal preparations or whole cell preparations? For any uncharacterized carcinogen, how could you use your knowledge to gain insights into metabolic bioactivation pathways using only subcellular fractionation techniques?**

**Additional Reading:**

Chemical Carcinogenesis, in "Casarett and Doull's Toxicology, The basic Science of Poisons. Pages 201-267 (CD Klaassen ed.) 5th Edition, McGraw Hill, San Francisco, CA.

Good web site:

<http://drnelson.utmem.edu/genesperspecies.html> (all the P450s, sequences relationships substrates etc.)