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Techniques for Assessment of Teratogenic Effects: Developmental Enzyme Patterns

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Most studies designed for assessing teratogenic effects focus on only three of four types of final manifestations of abnormal development; namely intrauterine death, malformations, and growth retardation. Developmental toxicity evaluations generally do not include functional deficits. Current techniques are inadequate for assessing functional capability during perinatal development, and there is a need for improved measures. Thus, measurement of developmental enzyme patterns is proposed as an approach that directly evaluates acquisition of metabolic competence of fundamental organ systems. During ontogenesis most organs acquire their full complement of enzyme activities in a programmed sequence which corresponds to attainment of complete functional capability. The usual alterations in enzyme activity that characterize these patterns occur at one of three time periods, namely, late fetal, early neonatal, or late suckling. Qualitative or quantitative changes in these patterns at any time by one or more key enzymes of a tissue could be indicative of developmental toxicity. Factors are outlined relating to consideration of developmental enzyme profiles as indices of maturation capable of reflecting the action of toxic agents. This presentation: (1) reviews the current state of the art for evaluating effects on development, (2) considers the applicability of enzyme patterns for biochemical assessment of development, (3) characterizes the target tissues and those metabolic pathways and/or specific enzymes most sensitive and adaptable to practical toxicity evaluations, and (4) describes the steps being taken to validate this system. The ultimate objective of this approach is to determine whether alterations in developmental enzyme profiles will provide a technique with improved capability for assessing developmental toxicity.

Introduction

There is a growing recognition of a need for more sensitive and meaningful methods for assessing the adverse effects of chemical agents on human health in general and on developing individuals in particular. This presentation will consider the use of enzyme profiles as indices of maturation, which, in turn, may reflect the action of toxic agents. This approach, measurement of enzyme patterns in developing tissues, directly evaluates the acquisition of metabolic competence by fundamental organ systems.

Most studies designed for assessing teratogenic effects, regardless of the type of agent, focus on only three of four final manifestations of abnormal development; namely, intrauterine or neonatal death, malformations, and growth retardation (*1*). For these studies the agents are given during major organogenesis, thereby producing exposures of the embryo. Evidence is accumulating which suggests that late exposure of the fetus and possibly even neonatal exposures to some agents may have deleterious developmental effects. Perinatal mortality is still a very real problem for infants throughout the world, and overt symptomology, such as prematurity with respiratory distress and/or jaundice, is seen frequently. Other effects may be less obvious. For instance, minimal brain dysfunction (MBD) in children may be an example of a subtle manifestation of a molecular, if not a cellular, lesion. While a chemically induced animal model for MBD has been developed in neonatal rats, its etiology in children is uncertain (*2*, *3*).

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Much of the work related to developmental toxicity has been directed toward assessing morphological and cyotogenetic changes and to a lesser extent, the behavioral aspects. We know very little regarding effects on biochemical systems, that is, the effects of agents upon the process of development after major organogenesis (*1, 4*). Unlike the gross structural manifestations of embryotoxicity, effects at the molecular level are not readily evident, and may be revealed only by special tests. A molecular or biochemical malformation may remain dormant for a period, then manifest itself later in life as a functional impariment, such as: behavioral disorder, learning disability, infertility, tumor susceptability, or immunological defect. Most organs during gestation are potential targets for insults that may produce cellular and/or molecular lesions with profound developmental disturbances for the individual.

Developmental toxicity evaluations generally do not include these types of functional deficits. Current techniques are inadequate for assessing functional capability during the perinatal period, and there is a need for improved measures (*4*). These problems require evaluation by whatever methods seem most appropriate. One approach is to focus on age-dependent changes in enzyme activity. Developing tissues contain distinctive enzyme complements that exhibit dramatic changes in their activity coinciding with the critical phases of the perinatal period. Greengard (*5*) has recommended that altered enzyme patterns be considered "biochemical malformations" that may serve as more subtle, sensitive and reliable indicators of perinatal dysfunction than observations of growth and survival rates. To date there have been few attempts to monitor effects of chemical agents on enzymes during development other than those involved in oxidative drug metabolism (*6, 7*). Significant impairment of neonatal rat liver and brain enzymes have been reported after irradiation in utero (*8-10*). Nevertheless, there have been no comprehensive attempts to fully explore and characterize this approach to developmental toxicity testing.

Several aspects must be considered in outlining any proposed model for relating alterations in functional maturation with altered enzyme profiles. This requirement has been considered as a series of specific questions that can be answered analytically and experimentally (Table 1). The first need is to consider the applicability of tissue enzyme patterns as biochemical indicators of functional capability. Second, it is necessary to characterize the presumptive target tissues and those pathways, especially their key enzymes, that are most suitable for practical toxicity evaluations. Third, the system still requires validation by initially testing classical physical and chemical teratogens before attempting to demonstrate broad applicability by testing putative agents.

Table 1. *Specific aims: to find answers to these questions* regarding the proposed system.

Applicability
How are perinatal studies important?
What relevance have they to humans?
What agents alter development?
When do the agents produce their effects?
Characterization
Which tissues are preferentially affected?
Which enzyme pathways are altered?
How should specific enzymes be selected for analysis?
Are qualitative or quantitative effects more important?
Validation: how can this approach be used to:
Ask environmentally relevant questions?
Design practical test procedures?
Evaluate environmentally related agents?
Predict human health hazards?

Applicability

The proposed system is based on the hypothesis that chemically induced changes in activity of perinatal enzyme systems during development can be detected and can serve as biochemical indicators of insult to developing animals.

During mammalian ontogenesis most organs acquire their full complement of enzyme activities in a programmed sequence which corresponds to attainment of complete functional capability (*4, 10-13*). Marked variations in many metabolic functions also occur during development. These changes are particularly dramatic at birth, with the abrupt transition from intrauterine to extrauterine environment and during the weaning period, when great variations in nutrition occur. These can be further divided into the late fetal, neonatal, and late suckling periods. A separate cluster of enzymes attains approximately adult levels of activity during each of these periods, coinciding with achievement of increased metabolic competence (*4, 13*).

To date, most perinatal studies involving enzyme patterns have used either normal or endocrine-modified animals to reveal the nature of the regulatory factors for gene expression during ontogenesis (*11*). Thus, the approach is relatively new to developmental toxicity evaluations.

Some toxic substances of current interest are aldrin/dieldrin, DDT, endrin, cadmium, lead, mercury, zinc, alkyl sulfonates, hexachlorophene, PCBs, poly(vinyl chloride), TCDD, ethanol, radionuclides, and in addition, malnutrition. While malnutrition is not an agent, it is one of the most common causes of perinatal problems (*14*). The other items listed represent examples of pesticides, industrial effluents, solvents, or surfactants with potential hazard as developmental toxicants. Studies on developmental toxicity historically have involved use of single agents administered under controlled conditions. Generally, the animals used were supplied with adequate to excess amounts of all essential nutrients and were maintained in a uniform environment. The general population does not live under such ideally controlled conditions. Actually, those most likely to experience inadvertant exposures to harmful agents are those most likely to suffer dietary imbalances, incontrolled drug usage, endocrine variations, or pathophysiological states that would increase their susceptability to an adverse reaction.

Test systems need to include some of these potentials for interactions along with usual procedures to determine their role in modifying the animal's response (*15, 16*). Identification of alterations in response following such exposures will be helpful in predicting the synergistic or protective actions of multiple influences with respect to developmental toxicity.

It has generally been accepted that the perinatal and neonatal periods are among the most sensitive stages of life. The lack of appropriate data on the response of these immature stages to exposure to toxic agents has prevented the full utilization and consideration of tissue enzyme ontogenesis as a test system.

Characterization

A number of factors justify the need to characterize developmental enzyme patterns. Of particular significance is the determination of which organ(s) represent(s) the major site(s) for adverse effects on maturation during different stages of development.

There is need to consider the distribution of selected enzymes in organs other than liver, including brain, lung, kidney, and intestine. However, the value of comparative organ studies will be influenced by the physicochemical properties of the agent, its probable route of entry, and its pharmacokinetics. Current studies are attempting to characterize effects on the developmental patterns of several enzymes in various tissues at different perinatal stages with classical physical and chemical agents. These types of studies also need to investigate the most commonly occurring and biologically significant pathways. Information gained from such studies can aid in predicting how the conceptus will respond to certain chemicals and whether this response is related to fetal disposition of toxic agents.

Analysis of enzyme patterns during hormonal or nutritional regulation and the control of gene expression during differentiation and especially in neoplastic tissues have been conducted by Weber et al. (*17-19*). These studies led to the identification of a number of key enzymes in various intermediary metabolism pathways that were considered to be the main targets of metabolic control. Some of the features that characterize them have recently been discussed (*18, 19*). A molecular correlation concept was developed to provide a means for studying gene expression at the molecular level and was based on the assumption that various cellular functions could be analyzed, correlated and understood in appropriate terms. Likewise, it is recognized that for an understanding of the regulation of the biochemical patterns and their correlation with appropriate biological characteristics of development, experimental efforts must concentrate on key enzymes and critical metabolic pathways.

Focusing on the key enzymes defined by Weber (*17-19*) precludes the necessity of assaying a larger proportion of the approximately 160 enzymes identified in rat liver alone (*20*). Rather, a quartet of key gluconeogenic enzymes and a trio of key glycolytic enzymes may adequately evaluate effects on carbohydrate metabolism (*21*). Similar groups of enzymes operating in purine, pyrimidine, DNA, ornithine, and cAMP metabolism have been identified as key enzymes (*21*). The ultimate selection of target tissue enzymes for testing thus requires consideration of characteristics of the presumed target tissue as well as those factors requiring specific knowledge, such as the probable action of the test agents. Such additional criteria for enzyme selection are: (1) low levels related to human disorders; (2) easily measured by standard assays; (3) distributed in more than one tissue; (4) active in soluble or bound form; (5) stability of appropriate preparations; (6) detectable in amniotic fluid.

Primarily, control of metabolism in mammals is achieved through modulation of activity and/or amount of regulatory enzymes. Thus, a

significant quantitative change would be one in which the shift from control activity levels differed by several orders of magnitude or a temporal deviation of more than one day was seen. Qualitative changes of note would be shifts in metabolic pathways from primarily synthetic to more degradative activity or shifts in isozyme patterns. Some of these aberrations might be permanent, but the stage of the conceptus at the time of exposure would influence the degree of response. Qualitative or quantitative changes in the enzyme patterns at any time by one or more key enzymes of a tissue could be indicative of developmental toxicity.

These studies should provide information concerning: (a) which organ or organs are most susceptable to alteration of constituent enzymes at different ages, (b) what pathways are most likely to be affected in a particular perinatal period, and (c) what is the inter- and intralitter variation in response of offspring to gestational insult?

Validation

A number of factors are being considered in the validation of this test system (Table 2). The insults selected for testing include salicylate and hydroxyurea (two classical chemical teratogens), radiation (a classical physical teratogen) and malnutrition (a common environmental condition). There is experimental evidence that both chemicals affect postnatal behavior in rats, when administered under the appropriate conditions that induce malformations, but given at lower levels (*22, 23*). Intrauterine exposure of rats and beagles to x- or γ-radiation has been reported to alter levels of several enzymes in liver and brain of neonatal animals (*8-10*). The potential for developmental problems following maternal malnutrition alone or in combination with such physical and chemical agents was discussed previously.

Our initial studies will be restricted to rats. As appropriate, other species may be included for their variations in degree of maturity of offspring at term and duration of gestation (*4, 24*). For the subsequent investigations, pharmacokinetic studies may be conducted concurrently with enzyme studies, depending on the agents selected for evaluation.

Test agents will be administered to the dam either during major organogenesis or during the late fetal period or, possibly, during lactation. Administration directly to the offspring during the first 48 hr postnatally will also be considered for some agents. Radiation will be given either as a single whole-body exposure or it will be limited to the exteriorized gravid uterus. Magnitude and duration of malnutrition will depend on the dietary components to be deleted, such as proteins, fats, carbohydrates, minerals, or vitamins.

A general outline of the proposed experimental design (Table 3) gives the major points regarding the exposure parameters and dosage schedule. Generally, dose levels tested will range from those toxic to the dam to those that appear to be without effect to the conceptus and will include at least one intermediate level. These studies will focus on the more subtle effects on biochemical and physiological processes that result from administration of low doses to immature animals as compared to adults. Control animals will receive an equal volume of vehicle or an appropriate sham procedure on the same gestation days as the treatments or no exposure. Body weights of dams will be monitored periodically throughout gestation. Generally, five to six litters will be included in each group.

As indicated in Table 2, "routine teratology" evaluations will be conducted on representative litters to correlate morphological events with the enzyme activity data. These evaluations will include: counting the implantation sites, noting the

Table 2. Factors considered for design of developmental toxicity evaluations.

Selection of test species
Ontogenic similarities to man
Pharmacokinetics of agent
Developmental stages for agent administration
Major organogenesis
Late fetal period
Early postnatal period
Exposure Parameters
At least three doses ranging from maximum tolerated to *no apparent effect*
Routes related to anticipated human exposure and properties of agent
Include both nontreated and vehicle controls
Dosage Schedule-Minimum Requirement
More than one developmental stage per dose
At least five animals per dose and stage
"Routine teratology" evaluation criteria
Toxicity: maternal weight change
Embryolethality: embryo resorptions and fetal deaths
Malformations: external, visceral, and skeletal
Growth retardation: fetal weight
Postnatal parameters: functional capacity
Growth rate and survival
Behavior and learning ability
Biochemical and physiological processes
Immunological determinants

Table 3. General experimental design.

Developmental stage	Gestational or postnatal day	Agent [a]		
		Salicylate	Hydroxyurea	Radiation
Fertilization	1			
	↓			
Major organogenesis	9			EXP [b]
	10	INJ [a]		
	11			
	12			
	13		INJ	
	↓			
Late fetal	18			
	19	INJ/SAC	INJ/SAC	EXP/SAC
	20	SAC [c]	SAC	SAC
	21	SAC [d]	SAC [d]	SAC [d]
	22	SAC	SAC	SAC
Postnatal	Birth			
	1	INJ	INJ	EXP
	2			
	3	SAC	SAC	SAC
	4	SAC	SAC	SAC
	5	SAC	SAC	SAC
	6	SAC	SAC	SAC
	7	SAC	SAC	SAC
	↓			
Preweaning	18	SAC	SAC	SAC
	19	SAC	SAC	SAC
	20	SAC	SAC	SAC
	21	SAC	SAC	SAC
	22	SAC	SAC	SAC

[a] INJ = Developmental period for agent administration at appropriate dosage levels.
[b] EXP = Developmental period for exposure to x or γ-irradiation.
[c] SAC = Developmental periods for enzyme analysis and/or measurement of other parameters.
[d] "Routine" teratology evaluations are performed on representative animals at gestation day 21.

general condition and viability of each conceptus, recording sex and weight for each live fetus, examining each pup for external malformations, and processing representative pups of each litter to detect visceral and/or skeletal alterations.

All enzyme measurements will be made by using standard procedures whenever possible, but modifications of some procedures may be needed. All enzyme assay methods will be verified under optimal conditions using purified enzymes, if commercially available. Generally, four to five consecutive ages of animals will be analyzed at each cluster. Thus, sudden fluctuations in enzyme activity can be detected and activity differences can be monitored throughout specific test periods.

There are a number of conceptual and technical errors that could lead to a lack of correlation between biochemical malformations and metabolic dysfunction: (a) marked changes in indicators of biochemical function may occur without affecting functional reserve capacity; (b) significant changes in enzyme activity may reflect reversible functional effects without permanent sequela,: (c) altered enzyme activity may reflect a generalized response to stress and not a specific response to the toxic agent; (d) marked species variations in enzymatic alterations may occur in response to a specific insult, especially if there are species differences in pharmacokinetics; (e) permanent effects on enzymes may depend on a specific time for the insult.

Thus, the effect of relevant gestational insults on perinatal enzyme patterns will be studied in reference to the following specific objectives. Developmental profiles of certain key enzymes will be traced in hepatic and some extrahepatic tissues at various time periods. Changes in enzyme activity profiles will be correlated with other functional indicators. Enzyme activities of corresponding maternal tissues will be measured simultaneous with those of their pups at the appropriate times. The importance of altered profiles to growth rates and survival for times up to and including sexual maturity will be investigated.

In summary, the ultimate objective of this approach is to determine whether alterations in enzyme profiles will provide a technique with improved capability for assessing developmental toxicity.

In October 1975, National Institute of Environmental Health Sciences (NIEHS) sponsored a conference entitled, "Enzyme Patterns in the Evalution of Developmental Toxicity" at the Research Triangle Park, N.C. The informative comments, observations, and discussions of the invited speakers and other participants were helpful in formulating my ideas. However, the statements given in this presentation are my own opinions, biases, and prejudices and are not intended to reflect those of the workshop participants. Special thanks go to Dr. Robert L. Dixon and other staff members at NIEHS for their contributions to the success of the workshop.

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