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The Action of a Dioxin-like Compound Upon Avian Thyroid and Vitamin A Homeostasis

by

Philip A. Spear

A thesis

presented to the University of Ottawa

in partial fulfillment of the

degree of Doctor of Philosophy in Biology

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ABSTRACT

Analysis of liver retinol and retinyl palmitate in the herring gull, Larus argentatus, revealed higher concentrations in a New Brunswick coastal colony than in colonies of the Great Lakes. Significant differences were also detected among Great Lakes colonies, and the lowest concentrations occurred in Lake Ontario gulls. The decreased retinoid concentrations corresponded with greater exposure to chlorinated dioxins and greater aryl hydrocarbon hydroxylase activity in the herring gulls reported previously. These results were duplicated under controlled conditions by injecting ring doves, Streptopelia risoria, with the dioxin analogue 3,4,3',4'-tetrachlorobiphenyl. The dioxin analogue caused a dose dependent decrease in liver retinol concentration which was significantly related to the induction of aryl hydrocarbon hydroxylase activity. In Great Lakes herring gulls, therefore, environmental exposure to dioxin-like compounds which induce aryl hydrocarbon hydroxylase may have caused the decrease in liver retinoids.

Further studies with ring doves demonstrated that the dioxin analogue caused imbalances in serum retinol. In fed doves exposed to the dioxin analogue, the serum retinol concentration increased; while in 24 h fasted exposed doves, serum retinol decreased. The decreased concentrations were quantitatively related to the induction of hepatic aryl hydrocarbon hydroxylase activity and uridine diphosphate glucuronyltransferase activity. These results were interpreted to mean that the induction of these enzymes

increased liver retinoid metabolism such that, in 24 h fasted birds, less retinol was available for release into the blood.

Ring doves were injected with the dioxin analogue one week before mating. Serum retinol concentrations at that time were significantly greater in exposed males and females compared with respective control groups. Exposed females laying viable eggs had significantly higher serum retinol concentrations than exposed females producing eggs which failed to develop to hatching. Embryotoxicity occurred between days 3 and 8 of incubation during which time yolk retinol and retinyl palmitate decreased significantly in both the normal eggs and embryotoxic eggs laid by exposed parents. These results indicate an effect upon retinoid metabolism/utilization in ovo.

In order to investigate effects upon the avian thyroid, ring doves were raised on low iodine diets which caused characteristic hyperplastic goiters. A single dose of the dioxin analogue reversed this condition within 7 days. The dioxin analogue produced large colloid goiters in the low iodine doves, and these goiters are qualitatively different from the hyperplastic-type commonly associated with low iodine intake and reported previously in Great Lakes fish and herring gulls. Therefore, dioxin-like compounds may not be responsible for the goiters observed in Great Lakes wildlife.

In poultry chicks receiving a low vitamin A -low iodine diet, the dioxin analogue caused growth rate reduction and a concomitant decrease in serum retinol. Changes in the thyroid status of poultry chicks was not related to growth effects.

On the basis of these results, a model of the toxic action of dioxin-like compounds is proposed.

RÉSUMÉ

L'analyse du rétinol et de la palmitate de rétinol dans le foie des mouettes, Larus argentatus a révélé des concentrations élevées dans une colonie marine du Nouveau-Brunswick comparativement aux colonies des Grands Lacs. Des différences significatives ont été discernées parmi les colonies des Grands Lacs et les concentrations les plus faibles furent décelées dans les mouettes du lac Ontario. La diminution des concentrations de vitamine A correspondait à l'exposition accrue aux dioxines chlorinées et à une plus grande activité d'hydroxylase d'hydrocarbures arylés signalées auparavant dans les mouettes. Ces résultats ont été reproduits dans le laboratoire. Des tourterelles à collier, Streptopelia risoria, furent injectées d'un analogue de dioxine, 3,4,3',4'-tetrachlorobiphenyl, ce qui produit une diminution de la concentration du rétinol hépatique. Cette diminution était reliée directement au dosage ainsi qu'à l'induction d'hydroxylase d'hydrocarbures arylés. Chez les mouettes, donc, l'exposition environnementale aux composés apparentés aux dioxines qui provoquent l'induction d'hydroxylase d'hydrocarbures arylés peut être responsable de la réduction de vitamine A dans le foie.

Des études ultérieures utilisant des touterelles ont démontré que l'analogue de dioxine était responsable du déséquilibre de rétinol sanguin. Parmi les tourterelles traitées à l'analogue de dioxine, on a constaté une hausse du niveau de rétinol sanguin chez les oiseaux nourris, mais une diminution de rétinol sanguin chez

les oiseaux jeûnant pendant 24 h. La concentration de rétinol hépatique a enregistré une diminution relative à l'induction des enzymes microsomaux hépatiques. Ces résultats indiquent que l'induction de ces enzymes a augmenté le métabolisme de la vitamine A hépatique de sorte que, chez les oiseaux jeûnant pendant 24 h, une quantité moindre de rétinol pouvait être transférée à la circulation sanguine.

Une semaine avant d'être accouplées, des tourterelles à collier furent injectées d'un analogue de dioxine et subirent une analyse sanguine. Comparativement aux groupes témoins respectifs, des hausses de rétinol sanguin significatives furent enregistrées chez les males et les femelles traités. Les femelles traitées à l'analogue de dioxine se répartissent en deux groupes; celles dont les oeufs féconds se développèrent normallement affichaient une hausse de rétinol sanguin significative comparativement aux femelles dont les oeufs féconds ne se développèrent pas jusqu'à éclosion. La mortalité parmi les embryons est survenue entre le troisième et le huitième jour d'incubation. C'est durant cette même période qu'une baisse significative de rétinol et de palmitate de rétinol fût enregistrée dans le jaune d'oeufs normaux et anormaux pondus par des parents traités à l'analogue de dioxine. Ces résultats démontrent qu'un effet se produit sur le métabolisme/ utilisation de la vitamine A in ovo.

Afin d'étudier les effets sur la glande thyroïde, des tourterelles à collier furent soumises à des rations alimentaires à teneur iodique réduite. Il en résulta des goitres hyperplastiques caractéristiques d'une carence en iode. Par la suite un seul

dosage de l'analogue de dioxine suffit à produire une réversion de cette condition en moins de sept jours. Chez les tourterelles dont la nourriture était faible en iode, l'analogue de dioxine produit de larges goitres colloïdaux. Ces goitres sont qualitativement différents du type hyperplastique communément associé à une carence en iode et déjà signalé chez les mouettes et les poissons des Grands Lacs. Il est donc possible que les composés apparentés aux dioxines ne soient pas responsables des goitres observés dans la faune des Grands Lacs.

Chez les gallinacés immatures soumis à une ration alimentaire faible en vitamine A et en iode, l'analogue de dioxine produit une réduction du taux de croissance accompagnée d'une réduction du rétinol sanguin. Les changements observés dans la condition de la thyroïde n'étaient pas reliés aux effets sur la croissance.

Fondé sur ces résultats, un model de l'action toxique des composés apparentés aux dioxines est proposé.

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CONTENTS

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Name	Philip Alfred Spear
Date of birth	March 15, 1950, Montréal
marital status	married
children	Natalie, born January 29, 1979
	Michelle, born January 29, 1979

EDUCATION

Concordia University, Montreal, Faculty of Arts, 1968-1970,
Majors: English and Religion

Carleton University, Ottawa, Special Studies, 1970-1972,
Various courses

Concordia University, Montréal, Faculty of Science 1972-1977,
Major: Biology
Degrees: B.Sc. 1974
M.Sc. 1977, supervisor: Dr. Perry, D. Anderson
Thesis title: "Copper Accumulation Kinetics and Lethal Tolerance in Relation to Fish Size".

University of Ottawa, Faculty of Science and Engineering, 1981-
Department of Biology
Doctoral Candidate, supervisors Dr. T.W. Moon
Dr. D.B. Peakall

EMPLOYMENT

1977/81 National Research Council of Canada, Environmental Secretariat.
duties: writing reviews of environmental contaminants (c.f. books on copper and zinc), assisting in the preparation of reviews (acid rain, cyanide, radioactivity)
supervisor: Dr. I. Hoffman

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AHH	aryl hydrocarbon hydroxylase
ANOVA	analysis of variance
AUFS	absorbance units full scale
cm	centimeter
da	day
DDA	2,2-bis (p-chlorophenyl) acetic acid
DDE	1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene
DDT	1,1,1-trichloro-2,2,bis (p-chlorophenyl) ethane
FTHI	free thyroid hormone index
g	gram
g-bw	gram body weight
HPLC	high pressure liquid chromatography
i.m.	intramuscular
i.p.	intraperitoneal
I.U.	international units
Kg	Kilogram
Kg-bw	kilogram body weight
KJ	kilojoule
M	molar
mg	milligram
µg	microgram
min	minute
ml	milliliter
µl	microliter
mm	millimeter
μ^3	cubic micrometer

N	normal
n	statistical sample number
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
ng	nanogram
nm	nanometer
PA	prealbumin
PBB	polybrominated biphenyls
PCB	polychlorinated biphenyls
pg	picogram
pmol	picomole
RBP	retinol-binding protein
SE	standard error
sp	species
T_3	triiodothyronine
T_4	thyroxine
TSH	thyrotropin
TT_3	total triiodothyronine
TT_4	total thyroxine
UDP-GT	uridine diphosphate glucuronyltransferase
v/v	volume per volume
w/v	weight per volume
2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
2,3,7,8-TCDD	2,3,7,8-tetrachlorodibenzodioxin
3,4,3',4'-TCBP	3,4,3'4'-tetrachlorobiphenyl

CHAPTER 1

INTRODUCTION AND THEORY

A field naturalist made the following observations concerning changes to bird populations near Kingston, Ontario, in the early 1970's:

> ...there are recent losses among several of our fairly common summer resident species. These may be of a more serious nature than the decreases up to fifteen years ago. All of these are, in part at least, fish-eating birds and include the Bald Eagle, Osprey, Double-crested Cormorant, Black-Crowned Night Heron, Caspian Common and Black Terns. Persecution and illegal shooting may be partly responsible for the decrease in cormorants and eagles but pesticide residues in the food of all of them are responsible for the very poor rates of breeding success. ...Herring and Ring-billed Gulls may also be on the downward path but not all the evidence for this is yet in (Quilliam, 1973).

Detailed investigations of herring gull colonies in the eastern basin of Lake Ontario were later to show phenomenally high accumulations of environmental contaminants, drastic reproductive failures, and congenital deformities. On a global scale, Lake Ontario and certain other areas of the Great Lakes became prime examples of the effects of chemical pollution on wildlife. Continued monitoring of the gulls since the early 1970's has demonstrated a steady decline in both the tissue residues of toxic chemicals and the extent of detrimental effects.

The overall objective of the research presented in this thesis

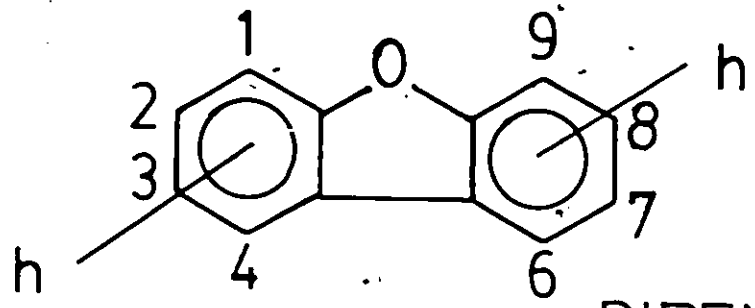
is the examination of mechanisms by which predominant environmental contaminants may affect wild bird populations of the Great Lakes area. In essence, the questions asked are fundamental to toxic mechanisms associated with a broad range of contaminants - the polyhalogenated aromatic hydrocarbons and are not necessarily limited to a particular class of organism. In this respect, the problem addressed is universal.

The species chosen as the model for environmental exposure to diverse contaminants is the herring gull, Larus argentatus, belonging to the order Charadriiformes. The herring gull was noted as a resident of the Great Lakes and east coast of Canada in Audubon's writings of 1840-1844 (Audubon, 1967). More than 90% of adult herring gulls of the Great Lakes region overwinter in the vicinity of their breeding colonies, while a small number migrate to the open waters of Lakes Michigan and Erie returning to breed in the early spring (Moore, 1976). Recruitment from Atlantic colonies to the Great Lakes is negligible (Kadlec and Drury, 1968). Thus, contaminant levels in herring gulls and their eggs tend to reflect geographical differences in exposure between Great Lakes colonies (Peakall et al., 1978; Mineau et al., 1984). The primary route of exposure is considered to be the consumption of fish and the associated ingestion of contaminants present in fish lipids. The herring gull is not strictly piscivorous, but relies upon alewives, Alosa pseudoharengus, and smelt, Osmerus mordax, as major food sources (Hunt, 1972; Jarvis and Southern, 1976; Allan, 1978). In order to understand effects occurring in wild bird populations, gull tissues were analysed. However, maintaining gulls under controlled conditions in captivity is not feasible and therefore

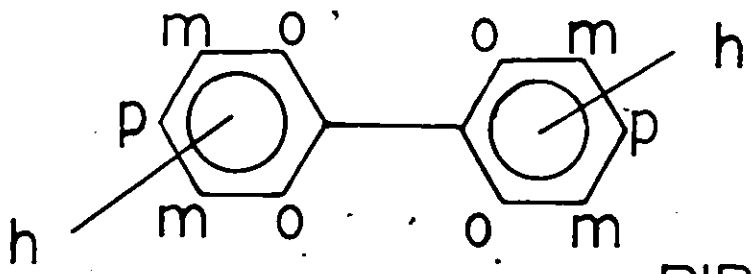
this species was deemed inappropriate for detailed studies of toxic mechanisms. The aviary species selected for investigation are the poultry chick, Gallus gallus, and the ring dove, Streptopelia risoria. The poultry chick, a descendent of the Asian wild jungle fowl, was noted by a companion of Magellan in 1521 to have been domesticated on South Pacific islands (Ball, 1933; Long, 1981), and has been raised in captivity for at least 4 centuries. This Galliformes is precoccial like the herring gull. The young achieve an advanced stage of development upon hatching and within a few days are independent with respect to locomotion, feeding and thermoregulation. Both the gull and poultry chick grow rapidly to a relatively large, near-adult size, i.e. approximately 1.5 Kg. Therefore, the poultry chick was specifically chosen in this study to investigate growth effects.

The ring dove is a domesticated Columbiformes which developed from the African collared dove, S. roseogrise (Goodwin, 1977). The ring dove is well-suited to the aviary as it has a small body size, i.e. 140-160g for adults, and a relatively short generation time of 6-8 months. Doves are altricial; the newly-hatched young are unable to walk and for a period of weeks are completely dependent upon parental care for food and thermoregulation. The reproductive cycle of the ring dove has been studied in detail (c.f. Lehrman, 1964). Previous investigations have established that the ring dove is an excellent species for reproduction studies involving environmental toxicants (c.f. Peakall and Peakall, 1973). Some advantages of using doves are the facility of maintaining breeding stocks, distinct behavioural patterns in courtship, uniform time of

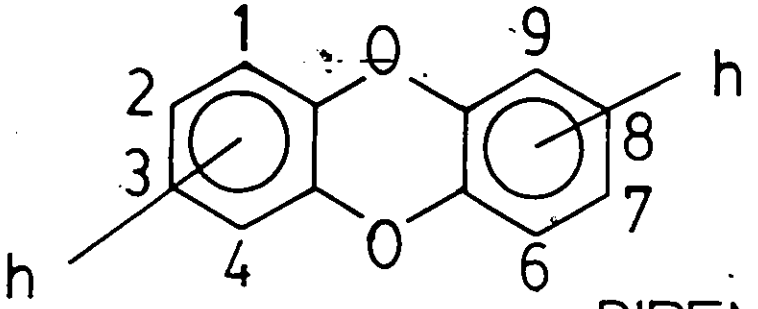
Fig. 1 Basic molecular structures of dibenzofuran, biphenyl, and dibenzodioxin molecules. Halogenation (h) positions are indicated numerically or as ortho (o), meta (m), and para (p).



DIBENZOFURAN



BIPHENYL



DIBENZODIOXIN

laying, and the inducibility of frequent breeding cycles throughout the year.

The present research pertains to a broad class of environmental contaminants that includes the highly toxic dibenzodioxins, dibenzofurans, biphenyls, and other stereochemically similar compounds. Representative dicyclic structures are illustrated in Figure 1. Taking into consideration the permutations of chlorine substitution on these three basic molecules, over 300 individual compounds are possible. The physical, chemical, and biological properties of the individual compounds are strongly influenced by both the amount and the specific position of halogen substituents.

Polychlorinated biphenyls (PCBs) were intended for use primarily in heat exchangers and electrical equipment due to their stability, heat capacity, and dielectric properties. Chlorinated dibenzofurans were unknowingly produced at very small yields in the commercial PCB mixtures, and are formed when PCB-containing fluids are heated, as during the normal operation of electric transformer installations. PCB manufacturing was stopped in North America in 1977 as a result of environmental concerns, but thousands of tonnes are presently utilized in Canadian electrical equipment. Chlorinated dibenzodioxins and dibenzofurans are formed as contaminants in the industrial production of chlorinated phenolic compounds and their derivatives. The commercial products penta- and tetrachlorophenol are used primarily as preservatives in the lumber and tanning industries, as well as in retail paints and stains. Derivatives of phenolic compounds include the widely used

phenoxy herbicides 2,4-dichlorophenoxyacetic acid (2,4,-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Another derivative, hexachlorophene, is manufactured from trichlorophenol and has applications as an agricultural disinfectant, as well as a bactericide and fungicide in cosmetic products.

Releases to the environment are associated with all aspects of commercial and industrial operations - manufacture, transportation, use, waste storage, and waste treatment. A second major source of furan and dioxin emissions is the incineration of municipal refuse and industrial wastes. The global contamination by PCB is well documented, while less is known of furans and dioxins because problems in chemical analysis have hampered monitoring initiatives. However, recent studies have demonstrated the ubiquity of polychlorinated dibenzofurans and dioxins in the environment, and relatively high concentrations are found in aquatic sediments and wildlife near known sources of emission (Ryan et al., 1985; Stalling et al., 1985).

The pathways and dynamics of these compounds in the environment are determined largely by their lipophilicity which tends to increase with increasing halogen substitution. For instance, the partitioning between aqueous and lipid/organic compartments, whether they be in the water column of a lake or within an organism is expected to vary according to the extent of chlorine substitution (NRCC, 1981). Rates of metabolism also favour faster body clearance of the less-halogenated compounds. These factors may be responsible for the greater accumulation of the higher chlorinated dioxins in Canadian human adipose tissue

(Ryan et al., 1985) as well as the relationship between whole body lipid content and interspecific differences in dioxin accumulation by fish (Ryan et al., 1984). In the case of PCB exposure of the white Carneau pigeon (species not given), few hepta- or hexachlorinated biphenyls were metabolized during ingestion and early tissue distribution, while most tetra- and pentachlorinated biphenyls were metabolized (de Freitas and Norstrom, 1974).

Generally, where the same extent of halogenation occurs in several homologues, e.g. several tetrachlorinated dioxins, the rate of metabolism and clearance is greater for the less-toxic compounds. Those dioxin and furan compounds which are readily metabolized tend to have adjacent (vicinal) unsubstituted positions (Rappe et al., 1979). Adjacent hydrogens presumably facilitate enzymatic detoxication in which a functional group, usually a hydroxy radical, is added to the present molecule. The result of such differential metabolism is that the more-toxic dioxin and furan compounds within a homologue grouping - i.e., the compounds having 2,3,7,8-substitutions - are found to comprise a greater proportion of the body burden (Rappe et al., 1979; Ryan et al., 1985).

Certain of the chlorinated dibenzodioxin and furans are the most toxic xenobiotic, or man-made, compounds in existence. The toxic potency varies between individual compounds by at least 8 orders of magnitude. For instance, in chronic mammalian testing, 1-10 ng/kg-bw/da 2,3,7,8-tetrachlorodibenzo-p-dioxin and 3 X 10^9 ng/kg-bw/da 1,3,6,8-tetrachlorodibenzo-p-dioxin were determined to be concentrations just below threshold for the elicitation of

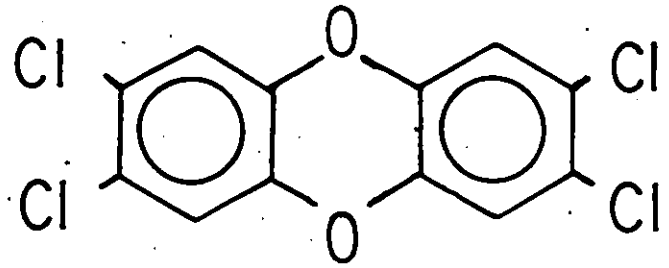
detrimental effects (Kociba et al., 1978; Kamata, 1983). A molecular mechanism has been investigated over the last decade which accounts for the enormous range in toxic potency. At the present time, it is thought that high affinity binding of the toxicant to a specific nuclear protein precedes genetic transcriptional and translational events. A number of enzymes are thus induced or repressed in a dose-dependent manner altering the metabolism of endogenous and exogenous compounds (Poland et al., 1979; Okey and Nebert, 1982). Relationships between the changes in enzyme activity and the characteristic symptoms of toxicity are incompletely understood, although the induced activity of aryl hydrocarbon hydroxylase (AHH) has been correlated with receptor affinity and gross physiological effects (Poland et al., 1976, 1979; Bradlaw and Casterline, 1979; Poland and Glover, 1980; Bandiera et al., 1982). AHH activity is generally accepted as a quantitative representation of the so-called cytochrome "P_{448}" monooxygenase system upon which it is dependent.

Structure-activity criteria have been developed for binding to the nuclear receptor and AHH induction (Sawyer and Safe, 1982; Bandiera et al., 1982, 1984; Safe et al., 1985). Owing to their rigid, planar configuration, the dibenzodioxin and dibenzofuran compounds have a higher affinity for the protein receptor than do equivalent biphenyl compounds which tend to rotate slightly about the central C-C bond (see Fig. 1 for molecular structures and nomenclature). Affinity is further influenced by halogen substitution. In the case of biphenyls, compounds which are not substituted in both para positions and at least two meta positions

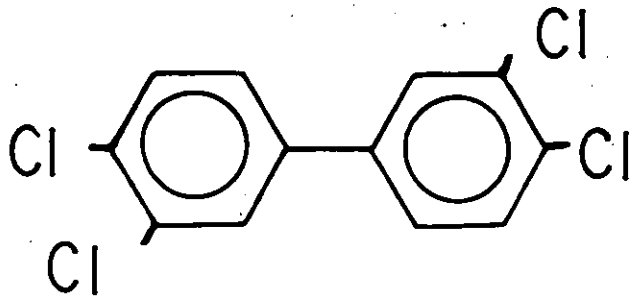
will not bind to the receptor, while ortho substitutions cause a moderate decrease in affinity. For dibenzodioxins and dibenzofurans, substitution at the four lateral ring positions (i.e. positions 2,3,7,8) gives maximum AHH induction and, in general, compounds without substitution at three lateral ring positions will not induce AHH. Substitutions at non-lateral ring positions tend to decrease induction potency. These criteria explain, for example, the tremendous difference in toxicity between the 2,3,7,8- and 1,3,6,8- congeners of tetrachlorodibenzodioxin mentioned earlier.

The compound 3,4,3',4'-tetrachlorobiphenyl (3,4,3',4'-TCBP) was chosen to investigate the effects of dioxin-like polyhalogenated aromatic hydrocarbons in the present research. It is analogous to the 2,3,7,8-congeners of dioxin and furan with respect to the positions of the chlorine groups (Fig. 2), specific binding to the nuclear "dioxin" receptor protein, and the induction of AHH (Yoshimura et al., 1979; Poland and Glover, 1980; McKinney and Singh, 1981; Okey and Nebert, 1982; Safe et al., 1982). The 3,4,3',4'-TCBP isomer of PCB was present at 2 μg/g in Japanese Yusho oil which poisoned hundreds of people in 1968 (Kuratsune et al., 1972; Trotter et al., 1982), and has been qualitatively identified in environmental samples collected from the Great Lakes area of North America (Smith and Johnson, 1983). Due to reasons mentioned above, the PCB isomer is approximately 1000 times less toxic than its dioxin and furan counterparts, and can be handled safely in conventional laboratories.

Fig. 2 Comparison of molecular structures of the highly toxic 2,3,7,8-tetrachlorodibenzodioxin and its analogue used in the present experiments.



2,3,7,8-TETRACHLORODIBENZODIOXIN



3,4,3',4'-TETRACHLOROBIPHENYL

Whereas much progress had been made in terms of the molecular mode of action and structure-activity relationships, what has eluded researchers is the mechanistic relationship between enzyme induction and principle toxic effects. Possible mechanisms between AHH induction and overt signs of toxicity were comprehensively reviewed by Parkinson and Safe (1981). They noted that many endogenous compounds are normally metabolized by monooxygenases not unlike the cytochrome "P_{448}" system which is integral to AHH function. Many aspects of toxicosis could be explained by alterations in the metabolism of certain of these endogenous compounds, however these authors concluded: "Studies on the effects of halogenated aryl hydrocarbons on various biochemical processes (carbohydrate, protein, lipid, nucleic acid, vitamin, hormone and mineral metabolism) have not revealed a mechanism of toxicity".

As a point of departure toward the development of a general hypothesis , therefore, the gross signs and symptoms of exposure were assessed, rather than reassessing the biochemical effects. The most detailed observations have been made on humans. In the case of Yusho oil poisoning in Japan, the symptoms were acne-like skin eruptions, eyelid edema, conjunctival discharge, skin and nail pigmentation and hyperkeratosis (Kuratsune et al., 1972). Numerous other incidents have been reported in which humans have developed acne-like skin disorders and hyperkeratinization in addition to more debilitating effects (Oliver, 1975; Kimbrough et al., 1977; Hay, 1979; Pocchiari et al., 1979). The skin condition is known as "chloracne" and is clinically diagnostic for exposure to dioxin-

like, chlorinated aromatics. Chloracne is virtually indistinguishable from hyperkeratosis which develops in cases of vitamin A deficiency. Also, hypothyroidism was diagnosed in many exposure cases. When the principle signs and symptoms of human exposure are compared with those of vitamin A imbalance and hypothyroidism (Table 1), a close correlation is evident.

Laboratory studies with rodents have shown that the dietary content of vitamin A influences the toxicity of polybrominated biphenyls (PBBs) and PCBs (Innami et al., 1974; Darjono et al., 1983) as would be expected if these toxicants decreased the efficiency of vitamin A utilization. Numerous studies have demonstrated decreased hepatic vitamin A. In rats exposed to 2,3,7,8-tetrachlorodibenzodioxin, the hepatic vitamin A (retinol) concentration decreased while hepatic uridine diphosphate glucuronyltransferase (UDP-GT) increased indicating that retinol metabolism had been accelerated (Thunberg et al., 1980). Unfortunately, these investigators used a saponification technique which converted retinyl esters to retinol and the specific retinoids could not be distinguished analytically. Serum retinol increased, or became more variable with no significant change, following exposure to 2,3,7,8-tetrachlorodibenzodioxin (2,3,7,8-TCDD) (McConnell et al., 1978; Thunberg et al., 1979; Thunberg and Hakansson, 1983). PBB exposure decreased or did not alter serum retinol (Bernert et al., 1983; Darjono et al., 1983). When animals were maintained on low vitamin A diets, exposure to PBB decreased serum retinol (Darjono et al., 1983).

Rodent studies have consistently shown decreases in serum thyroxine concentration following exposure to 2,3,7,8-TCDD and PCB

Table 1: Compilation of signs and symptoms of human exposure to dioxin-like compounds and comparison to those of vitamin A imbalance or hypothyroidism.

Signs and symptoms of human exposure to dioxin-like polyhalogenated aromatic hydrocarbons (Nicholson and Moore, 1979; NRCC, 1981; Parkinson and Safe, 1981).	Occurrence of comparable symptoms associated with hypothyroidism and abnormal vitamin A nutriture (Mazzaferri, 1974; Underwood, 1984; Kamm et al., 1984)		
	hypo-vitaminosis	hyper-vitaminosis	hypo-thyroidism
chloracne	+		
hyperkeratinization of skin	+		+
dry skin		+	+
nail discoloration			
increased perspiration			+
hirsutism (rapidly growing, thick hair)	+		
eyelid edema			+
abnormalities of immune response	+		
susceptibility to infections	+		
growth reduction	+		+
weight loss		+	+
vomiting		+	+
hepatomegaly (enlarged liver)		+	
increased SGPT[a]		+	
increased SGOT[b]		+	
increased serum cholesterol		+	+
altered iron metabolism	+		+
porphyria cutanea tarda			
nonspecific articular symptoms		+	+
fatigue, lack of drive		+	+
somnolence		+	+
altered behaviour and personality			+
headache		+	+
loss of balance	+		
ataxia			+
numbness of extremities			+

[a] serum glutamic pyruvic transaminase
[b] serum glutamic oxaloacetic transaminase

(Bastomsky, 1974, 1977; Bastomsky and Murthy, 1976; Collins et al., 1977; Collins and Capen, 1980a, 1980b). Hepatic UDP-GT activity increases the rate of conjugation of thyroxine and facilitates biliary excretion (Bastomsky, 1977). Detailed electron microscopic observations of the thyroid gland were interpreted to mean that PCB interfered with colloid droplet-lysosome interactions (Collins and Capen, 1980b) implying decreased thyroid hormone production from thyroglobulin. Light microscopic studies have revealed a hyperplastic appearance of the rat thyroid gland following exposure to PCB, PBB or 2,3,7,8-TCDD (Gupta et al., 1973; Collins and Capen, 1980a, 1980b; Akoso et al., 1982). Thyroid follicles typically had reduced thyroglobin colloid and increased epithelial cell height which could have been the result of increased TSH (thyrotropin) release from the pituitary in response to low levels of serum thyroid hormones. Collins and Capen (1980b), however, noted subtle ultrastructural differences between thyroids from PCB-exposed rats and those receiving iodine deficient diets or injected with TSH.

No avian studies have been previously reported concerning the effects of dioxin-like polyhalogenated aromatic hydrocarbons upon vitamin A status. Decreased liver stores of vitamin A were found in rock doves, Columbia livia, exposed to 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane (DDT) (Jefferies and French, 1971) which is a contaminant distinct from the dioxin-like compounds. Extensive nutritional studies have established that vitamin A imbalances, i.e. hypervitaminosis A or hypovitaminosis A, are associated with a diversity of anomalies in birds. These include growth inhibition, loss of secondary sexual characteristics, decreased weight of the

testes, decreased gametogenesis in the male, visual impairment, delayed egg laying, decreased egg size, embryonic mortality, increased incubation time, decreased hatchability, and deformities of bone and cartilage (Moore, 1957; Thompson, 1970, 1976). The effects of chronic, low-level PCB exposure in birds (Roberts et al., 1978) are markedly similar to the nutritional vitamin A anomalies.

A histological assessment of herring gull thyroid glands revealed a very high incidence of hyperplastic goiter in the Great Lakes area compared with samples collected from the Bay of Fundy, New Brunswick (Moccia et al., 1985). As in the cases of rodents exposed to dioxin-like compounds, thyroid follicles in Great Lakes gulls were seen to have increased epithelial cell heights and less colloid than in thyroids of the marine coastal gulls. The histological profile resembled iodine deficiency, and the Great Lakes area is known to be iodine-poor. These investigators, therefore, stated that goitrogenic agents, possibly polyhalogenated hydrocarbons, and/or iodine deficiency may be the cause of the goiters.

Laboratory studies in which black-backed gulls, Larus fuscus, and guillemots, Uria aalge, were exposed to PCB have demonstrated the development of large-colloid goiters (Jefferies and Parslow, 1972, 1976). Such goiters are the histologic opposite to the hyperplastic type; the follicle epithelia appear flattened while colloid size increases. Functionally, the large colloid goiter is indicative of gland inactivity, whereas hyperfunctionality is consistent with the hyperplastic form (Astier, 1980). It should be

noted, however, that organochlorine contaminants other than dioxin-like compounds, e.g. DDT and dieldrin, elicited hyperplastic goiter in birds (Jefferies and French, 1969, 1971, 1972). Toxicological investigations into the effects of dioxin-like contaminants on avian species have not previously involved the measurement of serum thyroid hormones. One study has been reported which dealt with thyroid function in birds. Depending upon dose level, PCB was found to increase or decrease I^{125} uptake into thyroid glands of the bobwhite quail (species not given) (Hurst et al., 1974).

In a major review of organochlorine effects upon avian species, Jefferies (1975) concluded that "...most of the sublethal effects of organochlorine insecticides may be due to two main lesions on the thyroid producing hyper- and hypothyroidism". The two mechanisms eluded to by Jefferies are i) increased hepatic metabolism and biliary excretion of thyroxine which would decrease plasma hormone levels and evoke greater TSH release from the pituitary, and ii) interference of thyroid hormone binding to plasma carrier proteins. The mechanisms have not been specifically examined in birds, but were nonetheless extrapolated from mammalian studies with DDT and its metabolites.

The general hypothesis explored in the present research is that toxicity of the polyhalogenated aromatic hydrocarbons, and 3,4,3',4'-TCBP specifically, is elicited through alterations in thyroid and/or vitamin A homeostasis. The first approach is to determine whether natural populations of herring gull have incurred vitamin A effects. The work of Moccia et al. (1985) has already revealed thyroid anomalies in Great Lake gulls. Secondly,

fundamental mechanisms of toxicity are investigated using nutritional stressors which to some extent simulate environmental conditions. Finally, effects upon thyroid and vitamin A homeostasis are evaluated at different dose levels encompassing the threshold for reproductive impairment.

CHAPTER 2

MATERIALS AND METHODS

2.1 Use of semi-purified diets

2.1.1 Low iodine studies with ring doves

Ring doves, Streptopelia risoria, generously supplied by the Canadian Wildlife Service, were held under controlled aviary conditions of 19-23°C and 14L:10D photoperiod. Birds of approximately four weeks of age and weighing 80-100 g were removed from parental cages and raised for five months on semi-purified diets plus distilled-deionized water, ad libitum. The diets were formulated according to the standard reference diet for poultry (US, NRC 1977) with the exception that reagent grade NaCl replaced iodized salt. The antioxidant ethoxyquin was omitted to circumvent possible effects on hormone metabolism. Fresh diets were prepared monthly in order to minimize degradation in the absence of the preservative. The basic diet mixture was divided in two parts and supplemented with either the minimum iodine requirement, 300 μg/Kg, or a low concentration of iodine, 3 μg/Kg, as KI. Preliminary studies demonstrated normal growth and reproduction on the full complement, semi-purified diet.

2.1.2 Low iodine-low vitamin A studies with poultry chicks

Leghorn chicks, Gallus gallus, were maintained under controlled conditions of 19-21°C constant light and 40-48% relative humidity. The chicks were raised to 3 weeks of age on a practical diet (Lab Cage Layer Chow 5070, Ralston Purina Canada Inc., Longueuil, Québec) containing 10,000 µg/Kg of carotene provitamin A, and 1,260 µg/Kg iodine. The birds were then placed on a basic semi-purified diet which was deficient in vitamin A and iodine. After 10 days, the birds were randomly sorted into 3 dietary groups; i) semi-purified low vitamin A (i.e. basic diet plus 350 µg/Kg iodine and 750 I.U./Kg vitamin A palmitate); ii). semi-purified low vitamin A-low iodine (i.e. basic diet plus 70µg/Kg iodine and 750 I.U./Kg vitamin A palmitate); iii) practical diet (i.e. Lab Chow 5070). Chicks were maintained on the experimental diets for a 12-day period. The iodine concentration of 70 µg/Kg was chosen to approximate levels causing growth inhibition in chicks (Creek et al., 1957). The higher iodine value, i.e. 350 µg/Kg, is near the threshold for changes in histology of the thyroid gland (Creek et al., 1957), but is above the daily requirements of poultry (U.S. NRC, 1977). The vitamin A concentration of the semi-purified diets is 15% of the minimum recommended level (U.S. NRC, 1977).

The semi-purified diets were formulated according to a standard reference diet for poultry (U.S. NRC, 1977) with certain modifications. Stripped corn oil (U.S. Biochemical Corp., Cleveland, OH) was utilized instead of ordinary corn oil which contains substantial amounts of the provitamin β-carotene, and other

retinoids. Reagent grade NaCl replaced iodized salt. The antioxidant, ethoxyquin, was omitted due to possible effects on vitamin and hormone metabolism. To minimize degradation in the absence of the preservative, diets were mixed and pelletized 2 days before the experiment and stored in light-proof, moisture-proof containers at 10-15°C. Food and distilled-deionized water were supplied ad libitum. Chicks were maintained on the experimental diets for a 12-day period.

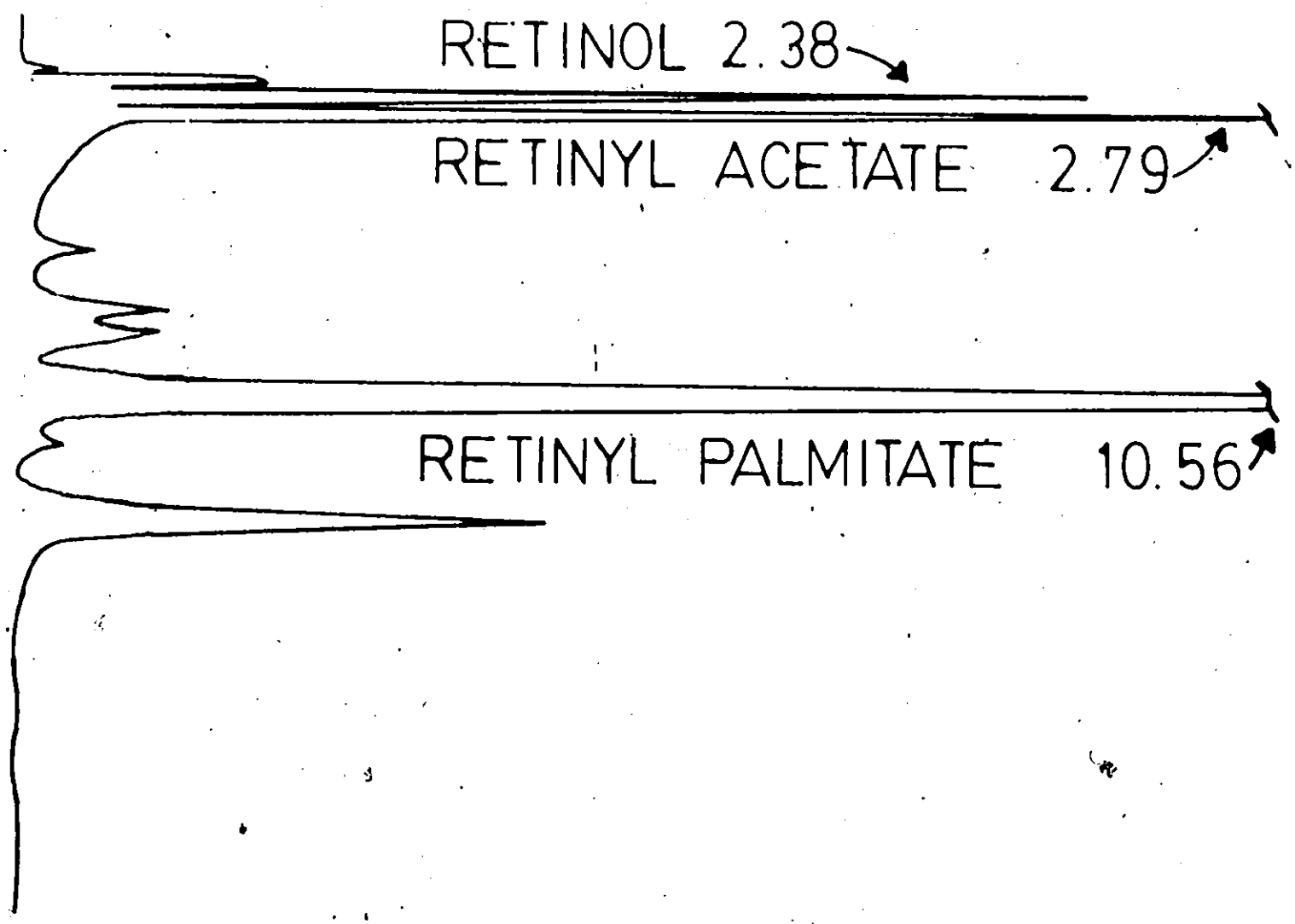
2.2 Retinoid analysis

Liver samples were specifically analysed for retinyl palmitate which is prominent among stored esterified forms, as well as retinol which is available for binding to the carrier protein and subsequent release into the blood.

Retinoids were extracted from liver tissue using a modification of the method of Ames et al. (1954). Partially thawed liver samples weighing 0.25 g were dehydrated by grinding with known amounts of anhydrous Na_2SO_4. The amount of dried powder calculated to contain 0.2 g of liver was spiked with 100 µl of retinyl acetate internal standard (400 µl/ml in methanol). Extraction was achieved by mixing for 10 min with 10 ml hexane. Following centrifugation (2000 x g for 2 min), a 500 µl aliquot was transferred to a half dram vial and evaporated to dryness at 60°C under a stream of nitrogen. The cooled residue was redissolved in 20 µl diethyl ether plus 180 µl methanol, and 10 µl samples were analyzed.

Retinoid analyses were conducted using high-performance liquid chromatography. The HPLC consisted of a model U6K injector, 6000A pump, precolumn filter and guard column, 30 cm x 3.9 mm C_{18} µBondapak analytical column and a model 441 absorbance detector set at 280 nm and 0.01 AUFS (Waters Assoc., Milford, MA). The solvent system was methanol-water (98:2) flowing at 2.0 ml/min. Peak areas were obtained using a model SP4100 computing integrator (Spectra-Physics, Santa Clara, CA). Retinoids were quantified from a standard curve of analyte and the peak ratio of analyte:retinyl acetate. The coefficient of variation was 3.6%, recovery was 92-100%, and the limit of detection was 2µg/g for retinol and 10µg/g for retinyl palmitate. A representative chromatogram of retinoid analysis in liver tissue is shown in Figure 3.

FIG. 3. Sample chromatogram of retinoids analysed in liver tissue of herring gull, Larus argentatus, collected from the Kent Island colony, New Brunswick. Retention times (min) are indicated for each vitamin A compound. Concentrations are 98 and 1238 μg/g retinol and retinyl palmitate, respectively.


RETINOL 2.38
RETINYL ACETATE 2.79
RETINYL PALMITATE 10.56

The yolks were analysed for retinoids (i.e. retinyl acetate, retinoic acid, retinaldehyde), which may accumulate as a result of metabolic disturbances, in addition to the forms usually detected (i.e. retinol, retinyl palmitate). Yolks were dissected from frozen eggs and dehydrated by grinding with 10 times the weight of anhydrous Na_2SO_4. Retinoids were extracted from 0.4 g of the yolk-Na_2SO_4 powder by mixing for 10 min with 2 ml tetrahydrofuran plus 2 ml n-hexane. After centrifuging for 5 min at 2000 x g, a 1 ml aliquot was transferred to a half dram vial and evaporated to dryness at 60°C under a gentle stream of nitrogen gas. The residue was redissolved in 100µl of reagent alcohol (90% ethanol, Fisher Sci., Fair Lawn, NJ) and 80 µl was injected into the HPLC. The HPLC system was as described above with the exception that 6000A and M-45 pumps were controlled by a model 660 solvent programmer (Waters Assoc., Milford, MA) and the absorbance detector was set at 340nm. The solvent system flowed at a constant rate of 2 ml/min and changed from acetonitrile (50%): 0.01M Na acetate (50%) to acetonitrile (100%) by linear mixing over a 12 min period. Peak areas were recorded on a model SP4100 computing integrator (Spectra-Physics, Santa Clara, CA) and quantitation was achieved with external standards (Sigma Chemical, St. Louis, MO). Recoveries were tested by spiking the yolk of a ring dove egg incubated to day 5 of development and frozen at -20°C until analysis (Table 2). Representative chromatograms of chemical standards and retinoids extracted from the yolk are shown in Figures 4 and 5.

Table 2. Recoveries of retinoids from yolk.

retinoid	retention time (min)	calculated limit of detection (ng injected)	(ng/egg)	range of spikes (ng/egg)	recovery (%)
retinoic acid	3.2	2	150	1125-4,500	91.0 ± 4.9[a]
retinol	10.5	2	150	525-2,100	88.7 ± 0.9
retinaldehyde	11.2	2	150	750-3,000	83.3 ± 3.3
retinyl acetate	12.2	2	150	750-3,000	86.0 ± 3.1
retinyl palmitate	22.9	5	375	750-3,000	101.7 ± 6.0

[a] mean ± SE; N = 5

Fig. 4. Sample chromatogram of retinoid chemical standards with HPLC conditions pertaining to yolk analysis. Retention times (min) are indicated for each vitamin A compound.

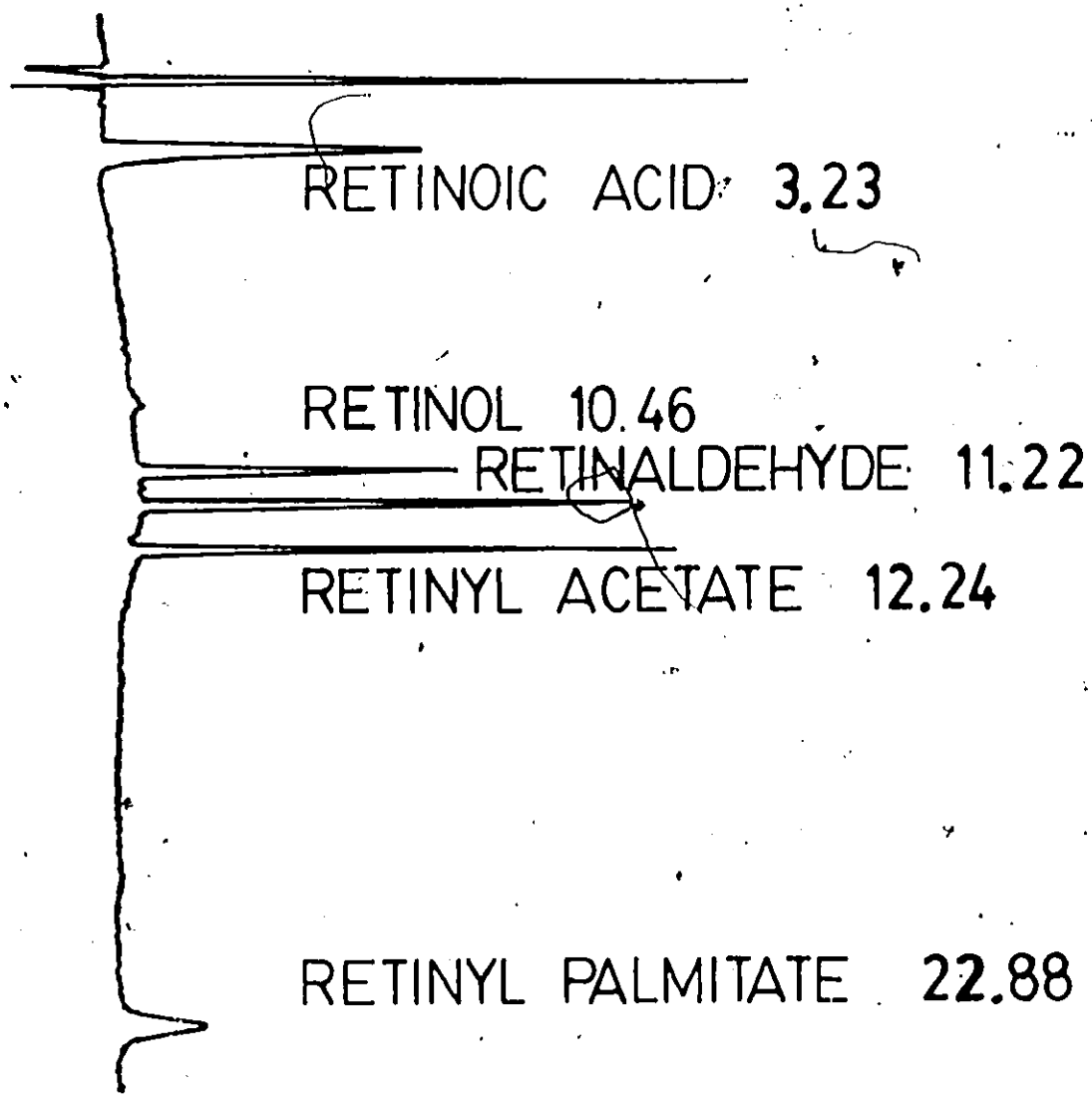
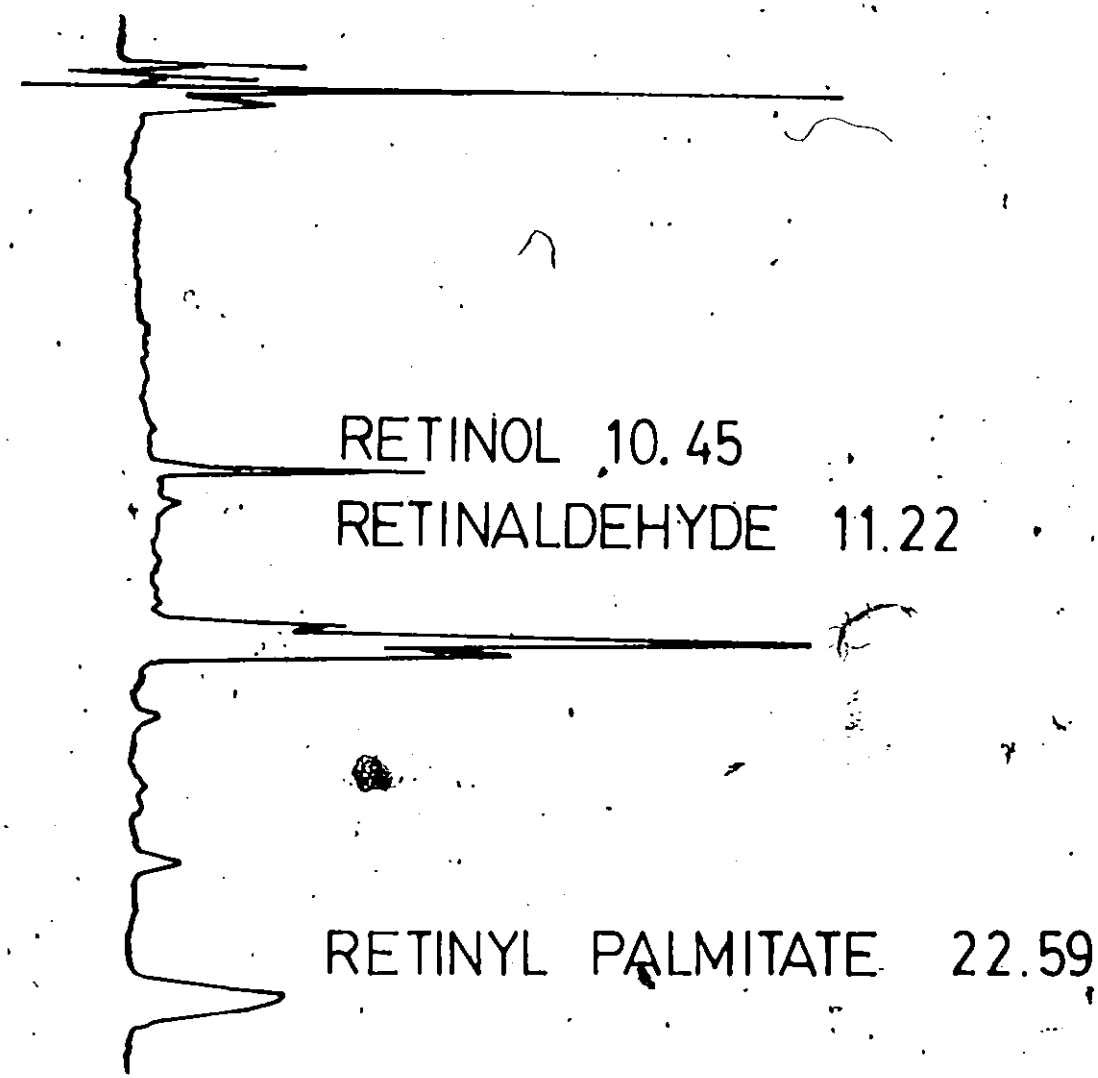

RETINOIC ACID 3.23
RETINOL 10.46
RETINALDEHYDE 11.22
RETINYL ACETATE 12.24
RETINYL PALMITATE 22.88

Fig. 5. Sample chromatogram of retinoids analysed in yolk of ring dove egg which had developed to 5 days incubation. Retention times (min) are indicated for each vitamin A compound.


RETINOL 10.45
RETINALDEHYDE 11.22
RETINYL PALMITATE 22.59

Serum retinol was quantified by reverse-phase HPLC according to the method of Bieri et al. (1979) with the following modifications. Extraction was achieved with 500 µl n-hexane giving a clear separation of aqueous and organic phases. A 200 µl aliquot of the organic phase was transfered to a half dram vial and evaporated to dryness at 60°C under a gentle stream of nitrogen gas. The cooled residue was redissolved in 10 µl diethyl ether followed by 100 µl of HPLC grade reagent alcohol (90% ethanol; Fisher Scientific, Fair Lawn, NJ). A 50 µl volume was injected into the HPLC system. All HPLC equipment and assay conditions were identical to those of Bieri et al., (1979). Peak areas were recorded on a model SP4100 computing integrator (Spectra Physics, Santa Clara, CA). The relative dose response (RDR) to retinyl palmitate administration was calculated as;

$$RDR = \frac{R_3 - Ro}{R_3} \times 100\%$$

where Ro is the serum retinol concentration after 24 h fasting, and R_3 is the serum retinol concentration 3 h after retinyl palmitate administration.

2.3 Thyroid hormone analysis

Total thyroxine (TT_4) and total triiodothyronine (TT_3) were determined with Quantimune radioimmunoassay kits (Bio Rad Laboratories, Mississauga, Ontario, Canada), and T_3 resin uptake was measured with a Bio-Ria (Montréal, Québec, Canada) radioimmunoassay kit. Standards provided with the TT_4 kit were diluted 50% with serum devoid of thyroid hormones in order to increase the sensitivity. Hormone standards consisted of six serum concentrations in the ranges 0-100 and 0-10 ng/ml for TT_4 and TT_3, respectively. The T_3 uptake standards were 25.6%, 31.9%, and 41.3%. A single RIA kit was used for each type of assay to circumvent possible inter-assay variation. The intra-assay coefficient of variation on ten replicate samples was 4.8%, 5.1%, and 5.9% for TT_4, TT_3, and T_3 uptake, respectively.

2.4 Porphyrin analysis

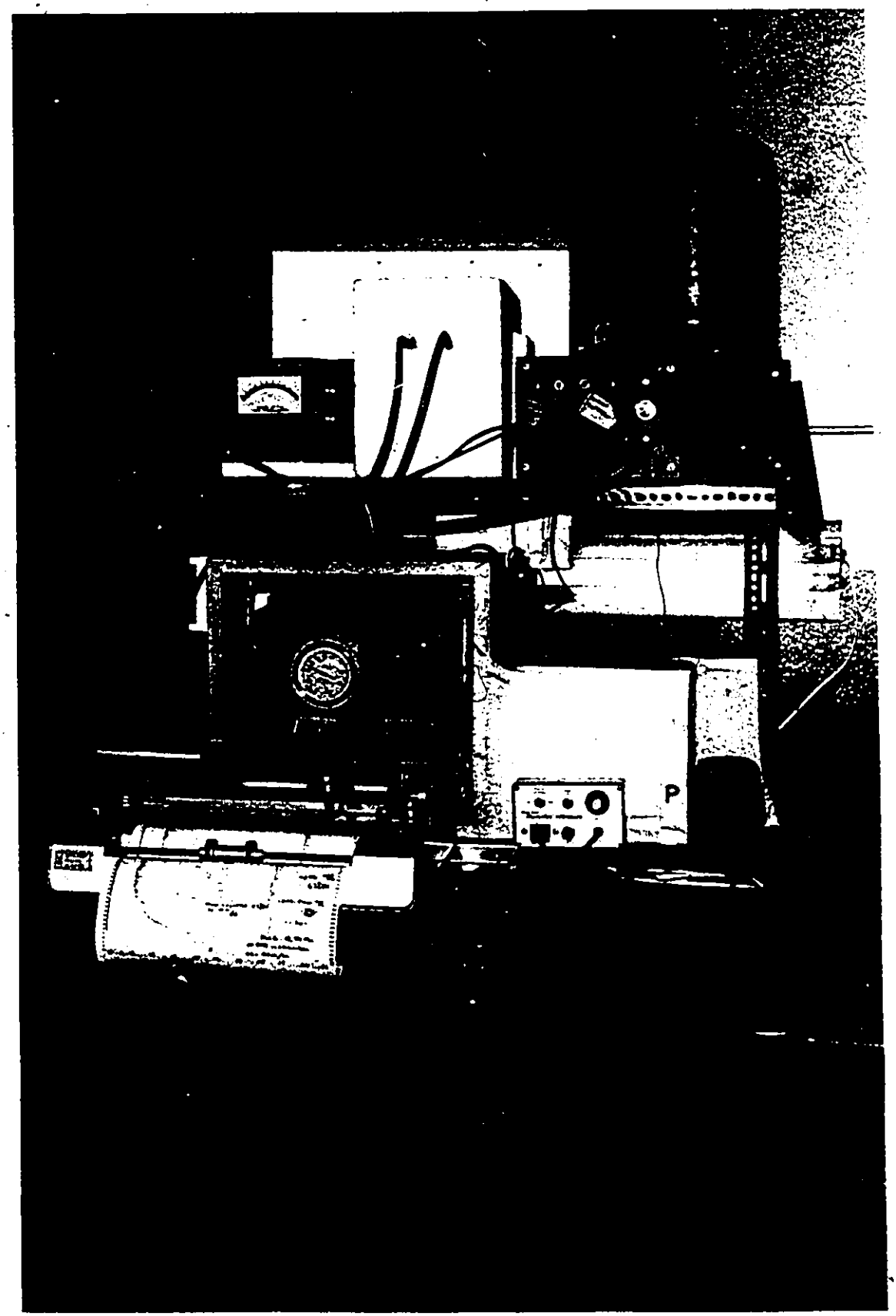
Uroporphyrin was extracted from liver tissue by the method of Abbritti and De Matteis (1971/72). Thawed tissue (1 g) was homogenized in 10 ml of 0.9 N perchloric acid-methanol (50:50). Following an 8 min centrifugation at approximately 2000 x g, the supernatant was decanted into 30 ml of distilled water. The pellet was re-extracted, centrifuged, and the supernatant diluted as before. The extracted porphyrins were concentrated on a SEP-PAK C_{18} cartridge (Waters Assoc., Milford, MA) eluted in 1 ml methanol, and passed through a microfilter. Uroporphyrin was specifically analysed using reverse phase HPLC with modifications to the method

of Ford et al. (1981). The HPLC system consisted of a Series 4 chromatograph, a 3 cm X 3.9 mm C_{18} (3 μ particle size) column, a LS4 fluorescence spectrophotometer (396 nm excitation; 624 nm emission) and a Sigma 15 recording integrator (Perkin-Elmer, Toronto, Ont.). The methanol-buffer (45:55) solvent system was programmed to change linearly over a 2 min period to methanol-buffer (95:5) which was maintained for 2.5 min. The buffer solution was 0.1 M sodium phosphate in doubly deionized-distilled water. The flow rate was held constant at 2 ml/min. Uroporphyrin external standards were used to calibrate the endogenous porphyrin concentration through a linear range of response. Uroporphyrin was 90-100% recovered by this method.

2.5 Calorimetry

The calorimeter consisted of a 32 X 32 X 60 cm stainless steel cage placed inside a thermally insulated chamber (Fig. 6). Windows permitted behavioral observations and the maintenance of a normal photoperiod. Metabolic heat produced by the birds was transferred to water flowing at 10-12 ml/min through copper tubing located in the chamber ceiling. Thermistor probes monitored the inflowing (i.e., approximately 12°C) and outflowing water and these temperatures were automatically recorded at 2 min intervals over a 24 h period. Food and water were supplied ad libitum and air was exchanged at 100 ml/min. The calorimeter was not adiabatic and consequently the efficiency varied from 62% to 145% depending upon

Fig. 6. Calorimeter used to determine metabolic rates.
I = insulated chamber
C = cage
R = recorder
T = telethermometer
S = temperature probe switch
B = constant temperature water bath
W = water reservoir
P = air circulating pump


P

room temperature, water flow rate, and heat production rate. All data were corrected to 100% efficiency. A group of three birds was tested simultaneously in a given trial, and two trials were conducted for both the control and exposed groups (i.e. N=2 independent trials per treatment). In the case of doves, data for the period 2:00 am to 6:00 am were taken to represent the standard metabolic rate, because the birds were in a post-absorptive, inactive state under thermoneutral conditions. Poultry chicks were tested under subdued lighting which induced roosting behavior.

2.6 Histology

The condition of the thyroid glands was assessed by light microscopy. The glands were excised, weighed, fixed in Bouin's solution, embedded in paraffin, and 4μ sections stained with hematoxylin and eosin. Colloid diameters were measured in all follicles of three sections randomly selected from each gland.

Samples of the thymus gland were fixed in Bouin's (8 h) embedded in paraffin, and 4μ sections stained with Harris hematoxylin and eosin Y. In order to estimate the number of lymphocytes per volume of thymus gland in a given bird, lymphocytes were counted in ten areas of the medulla which were randomly selected from each of 3 sections.

2.7 Enzyme assays

Liver microsomes were prepared by the method of Lucier *et al.*

(1973). Freshly excised livers were minced with scissors and submerged in ice-cold HEPES-KCl (20 mM HEPES, 0.15M KCl, pH 7.4) to give a 20% (w/v) mixture. The following procedures were all carried out at 0-4°C. The minced livers were homogenized with 6 strokes of a Potter-Elvehjem tissue homogenizer. Homogenates were centrifuged at 670 x g for 10 min. The resultant supernatant was centrifuged at 10,000 x g for 15 min, and the supernantant for the second spin centrifuged at 105,000 x g for 70 min. The resulting pellet was washed twice with HEPES-KCl and resuspended in 20 mM HEPES (pH 7.4) to give 2 ml of suspension per g liver tissue.

The activity of uridine diphosphate glucuronyltransferase (UDP-GT) was determined by a modification of Hollmann and Touster's (1962) procedure. A 3 ml aliquot of microsomal suspension was incubated for 5 min at 42°C with 60 µg of 10% (v/v) Triton X-100 (Sigma Chemical, St. Louis, MO) in 20 mM HEPES (pH 7.5). The detergent-treated microsomes (600 µl) were added to an incubation mixture pre-warmed to 42°C which contained 280 µl of 42.8 mM $MgCl_2$ 200 µl of UDP-glucuronic acid and 120 µl of 10 mM p-nitrophenol. The reaction was stopped 10 min later with 1 ml of chilled trichloroacetic acid (5% w/v), centrifuged for 10 min at approximately 2000 x g, and 1 ml of supernatant was added to 10 ml KOH (4.5% v/v). Blank tubes contained distilled water in place of glucuronic acid. Reaction and blank tubes were run in duplicate and the utilization of the p-nitrophenol substrate was measured as change in optical density at 405 nm. Preliminary experiments established that the reaction rate was constant for an initial 10 min period.

Aryl hydrocarbon hydroxylase activity was measured by the method of Yang and Kicha (1978). The microsomal suspension (not treated with Triton X-100) was diluted 200-fold in a pH 7.4 buffer consisting of 0.02 M HEPES, 5 mM $MgCl_2$, and 0.1 M EDTA. A 2 ml aliquot was placed in a cuvette and warmed to 42°C over a 5 min period. Then 20 µl of 0.1 mM benzo(a) pyrene was added and the mixture equilibrated for 2 min. The reaction was initiated by the addition of 0.2 µmol NADPH in 50 µl of HEPES. The quenching of fluorescence (387 nm excitation; 407 nm emission) was detected using a model 430 spectrofluorometer (Turner Assoc., Palo Alto, CA) equipped with a constant temperature cuvette holder. The change in fluorescence was constantly recorded on a Series 100 recorder (Fisher Sci., Fair Lawn, NJ). The initial reaction rate was estimated from the non-linear recording, and quantitation was achieved by a standard curve of benzo(a) pyrene in microsomal suspension. Microsomal protein was measured in the diluted suspension using the coomassie blue assay of Bio-Rad (Richmond, CA). All enzyme reactions were conducted within 5-7 h of sacrificing the doves.

CHAPTER 3

FIELD STUDIES WITH THE HERRING GULL

3.1 Rationale and experimental design

Comprehensive monitoring of herring gull eggs (c.f. Norstrom et al., 1982) and human adipose tissue (c.f. Ryan et al., 1985) has revealed that polychlorinated dibenzo-p-dioxins and dibenzofurans are ubiquitous contaminants in the environment. Geographic patterns of accumulation in North American wildlife species are consistent with regions of manufacture, use, and waste management practices associated with chlorinated phenolics (NRCC 1981; Stalling et al., 1985). Thyroid anomalies in herring gulls of the Great Lakes were characterized by Moccia et al. (1985). The present study was conducted to determine whether chemical pollutants have affected the vitamin A status of the herring gulls.

Adult herring gulls, Larus argentatus, were trapped on their nests in the spring of 1982 from four collection sites: i) Kent Island in the Bay of Fundy, New Brunswick, ii) Granite Island in Black Bay, Lake Superior, iii) Bellows Island in Grand Traverse Bay, Lake Michigan, and iv) Presquile in the eastern basin of Lake Ontario. The gulls were sacrificed by cervical dislocation and the livers were immediately excised, quick-frozen in liquid nitrogen and stored at -50°C until analysis for retinoids. Due to the small sample sizes, normality and homogeneity testing of the data were considered inappropriate. Therefore, comparison between collection sites was achieved non-parametrically by the Kruskal-Wallis 1-way ANOVA (Siegel, 1956).

Under controlled aviary conditions, juvenile ring doves, Streptopelia risoria, received single intraperitoneal (i.p.) injections of 10, 20, or 40 μg/g body weight of 3,4,3',4'-TCBP (Analabs, New Haven, CT) suspended in vitamin-stripped corn oil (U.S. Biochemical Corp., Cleveland, OH). The doves were sacrificed by CO_2 inhalation 4, 12, or 52 days later. The left liver lobes were quick-frozen in liquid nitrogen and stored at -90°C until analysis for vitamin A. Microsomes were prepared from the freshly-excised right lobes and assayed for AHH activity. Linear regressions of enzyme activities and retinoid concentrations were evaluated by Bartlett's three-group method for Model II regressions (Sokal and Rohlf, 1969).

3.2 Results

Retinoid concentrations in herring gulls varied between geographic locations being significantly greater ($p<0.005$) in New Brunswick gulls than in gulls of the Great Lakes (Table 3; Fig. 7). The values were also significantly different between Great Lakes colonies ($0.01<p<0.025$, retinol; $0.025<p<0.05$, retinyl palmitate). The Granite Island colony located on Lake Superior had the highest average retinoid concentrations, while the lowest average concentrations occurred in the Presquile colony on Lake Ontario. Values pertaining to the 1982 gull retinoid analyses are contrasted in Table 3 with the extent of environmental dioxin exposure as indicated by 2,3,7,8-TCDD in gull eggs collected in 1980 and 1983. The dioxin analyses indicate that gulls of the Lake Ontario colony endure a higher dioxin exposure than do gulls of Lake Michigan, Lake Superior, or New Brunswick.

In the case of ring doves exposed to 3,4,3',4'-TCBP, liver retinol concentrations ranged from 54 to 144 µg/g and the lowest levels occurred 12 days following exposure to the highest dose of the dioxin analogue (Table 4). Retinyl palmitate ranged from 932 to 2243 µg/g. Liver microsomal UDP-GT activity reached relatively high levels 52 days following the single injection of toxicant. At the highest dose, AHH activity apparently peaked before 52 days. Decreasing retinol concentration was significantly related to increasing AHH activity (Fig. 8). No other relationships between the retinoids and the inducible enzymes could be discerned from these data.

TABLE 3. Retinoid concentrations in the liver and 2,3,7,8-TCDD concentrations in eggs of herring gulls (Larus argentatus)

	Liver retinyl palmitate(μg/g)	Liver retinol (μg/g)	Egg 2,3,7,8-TCDD[a] (pg/g)	
Collection area	1982	1982	1980	1983
Lake Ontario	(Presquile colony)		(Presquile, Muggs Island, Scotch Bonnet Island, and Snake Island colonies)	(Snake Island colony)
	median 231	131	mean 59	90
	range 71-495	72-289	range 43-72	
	sample no. 17	17	sample no. 4	1
Lake Michigan	(Bellows Island colony)		(Big Sister Island colony)	(Gull Island colony)
	median 377	289	mean 9	10
	range 87-1342	77-941	range	
	sample no. 10	10	sample no. 1	1

Lake Superior		(Granite Island colony)		(Granite Island and Agawa Rock colonies)		(Agawa Rock colony)
	median	562	382	mean	11	13
	range	412-896	251-520	range	9-12	
	sample no.	4	4	sample no.	2	1
New Brunswick		(Kent Island colony)		(Kent Island colony)		
	median	1737	864	mean	3	
	range	141-5029	43-2012	range		
	sample no.	20	20	sample no.	1	

[a] data from NRCC 1981; Norstrom et al. 1982; Stalling et al. 1985; R.J. Norstrom, pers. commun.; each sample was a composite of 10 eggs representing a given colony.

FIG. 7. Retinol (hatched bars) and retinyl palmitate (open bars) concentrations in herring gull liver samples collected from colonies on the Great Lakes and the New Brunswick coast in 1982. Horizontal lines represent groups of colonies which were compared statistically. Significant differences within groups are shown.

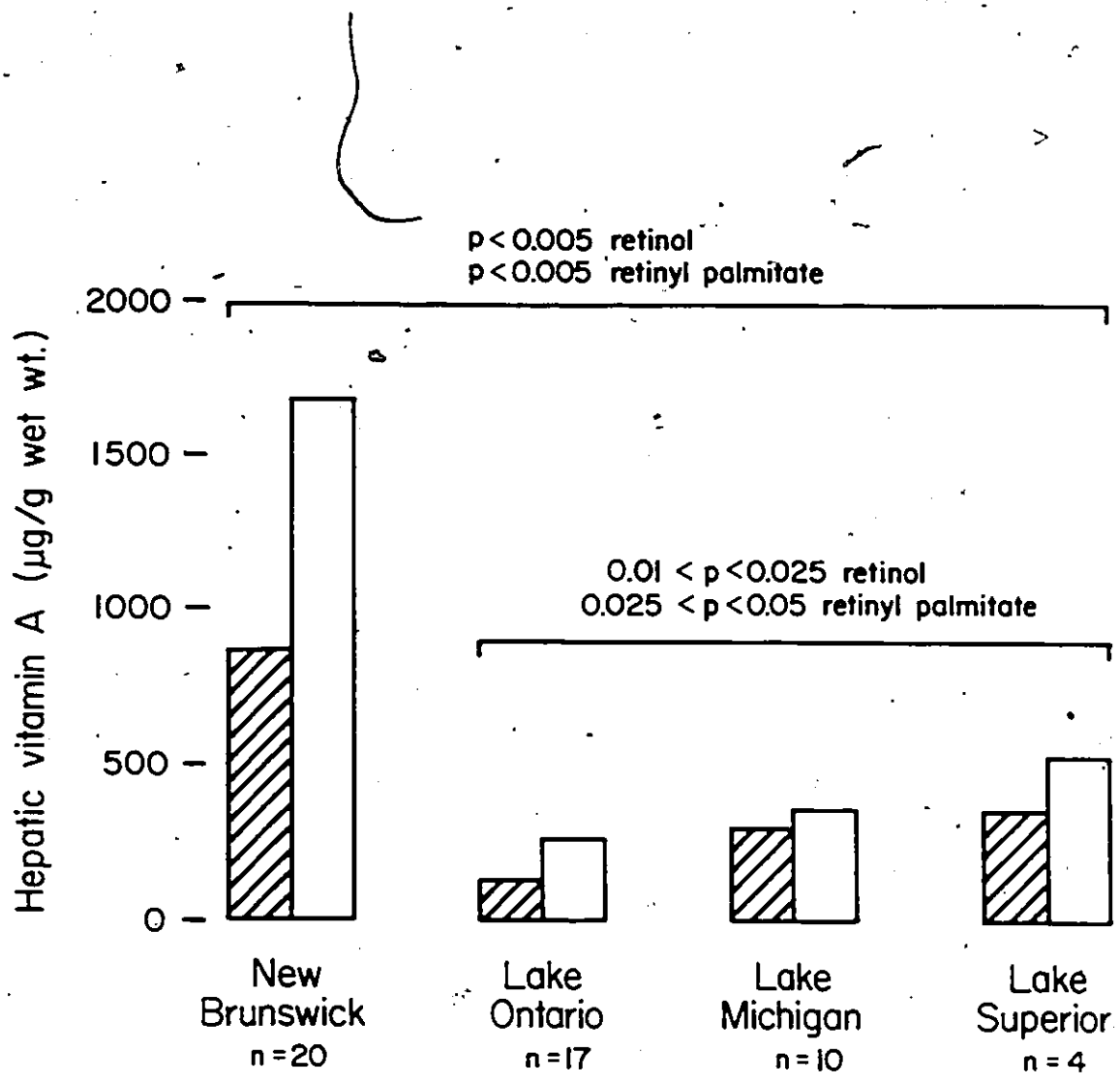

p < 0.005 retinol
p < 0.005 retinyl palmitate
2000
1500
1000
500
0
Hepatic vitamin A (µg/g wet wt.)
0.01 < p < 0.025 retinol
0.025 < p < 0.05 retinyl palmitate
New Brunswick
n = 20
Lake Ontario
n = 17
Lake Michigan
n = 10
Lake Superior
n = 4

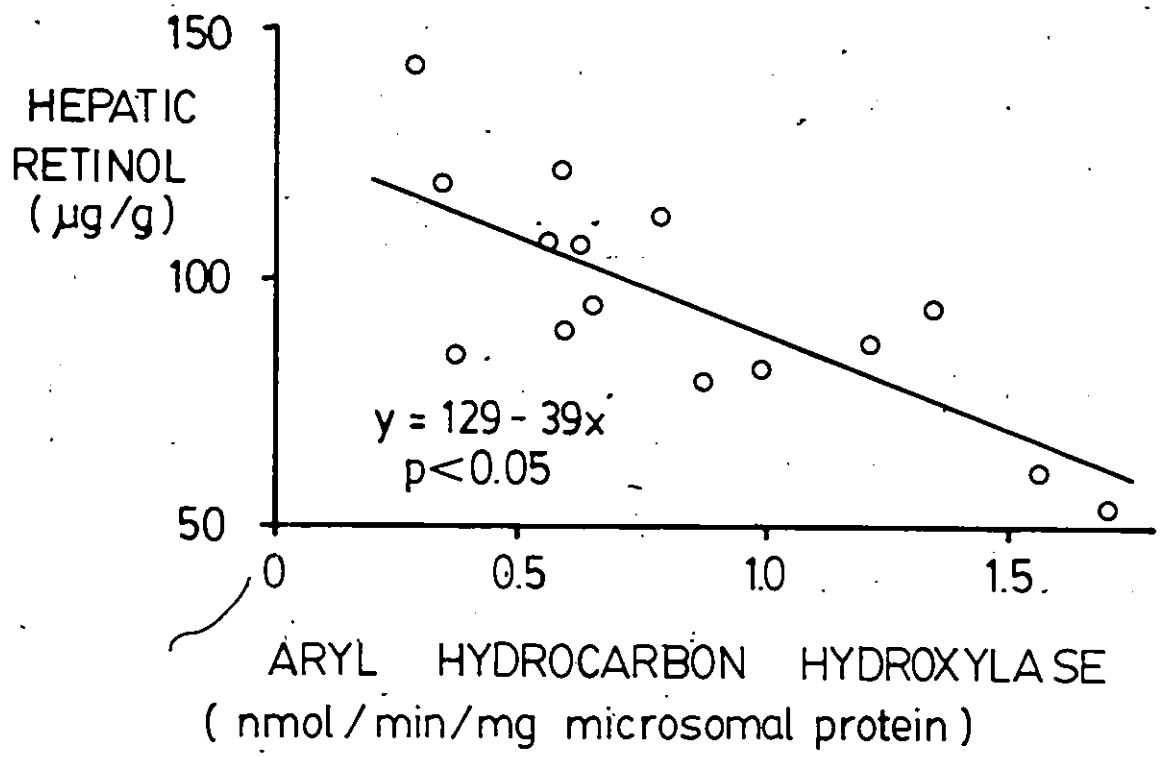
TABLE 4. Liver microsomal enzyme activities and retinoid concentrations in ring doves (Streptopelia risoria) exposed to the dioxin analogue 3,4,3',4'-tetrachlorobiphenyl

Dose injected (µg/g-bw)	Duration of exposure (da)	Retinyl palmitate (µg/g)	Retinol (µg/g)	UDP-GT[a] (nmol/mg protein/min)	AHH[b] (nmol/mg protein/min)
10	4	1320	84	0.125	0.375
10	4	1053	124	0.281	0.594
10	4	947	80	0.172	0.781
10	4	1617	90	0.244	0.594
20	52	1892	108	0.144	0.625
20	52	1279	144	0.156	0.281
40	4	1121	95	0.363	1.344
40	4	2243	88	0.338	1.219
40	4	1720	95	0.116	0.656
40	4	1596	82	0.384	1.000
40	12	1570	62	0.327	1.559
40	12	1614	54	0.421	1.704
40	52	2123	113	0.172	0.781
40	52	1471	120	0.672	0.344
40	52	932	108	0.538	0.563

[a] UDP-glucuronyltransferase (p-nitrophenol conjugation).

[b] Aryl hydrocarbon hydroxylase (benzo(a)pyrene hydroxylation).

Fig. 8. Regression of hepatic retinol concentration on aryl hydrocarbon hydroxylase activity in ring doves, Streptopelia risoria, dosed with 3,4,3',4'-tetrachlorobiphenyl. Coordinates are obtained from the data in Table 4.


150
HEPATIC
RETINOL
(μg/g)
100
50
y = 129 - 39x
p<0.05
0
0.5
1.0
1.5
ARYL HYDROCARBON HYDROXYLASE
(nmol / min / mg microsomal protein)

3.3 Discussion

Among vertebrates the predominant stored form of vitamin A is generally retinyl palmitate, and substantial amounts of this retinoid may accumulate in the liver (Thompson, 1976). A preliminary screening of avian tissues confirmed that the liver, and secondarily the kidney, contained the largest amounts of retinyl palmitate (Spear unpubl. data). Therefore, hepatic retinyl palmitate was specifically analyzed as an indicator of vitamin A reserves in the gulls. Liver retinol represents non-stored vitamin A which, under normal conditions, may either become esterified and stored or may become bound to a carrier protein and distributed to peripheral tissues (Goodman and Blaner, 1984). In certain diseases, e.g. protein-calorie malnutrition, peripheral vitamin A deficiency may develop despite adequate liver reserves (c.f. Underwood, 1974). Hepatic retinol was therefore analyzed to indicate anomalies which may occur independently of stored vitamin A.

Under normal conditions, liver retinoid concentrations tend to reflect vitamin availability in food (Underwood, 1974; Thompson, 1976). The relatively high concentrations in the New Brunswick colony (Table 3) presumably arise from high retinoid and carotenoid concentrations in marine fish consumed by the gulls, whereas Great Lakes gulls may eat fish containing lower concentrations of retinoids and carotenoids.

The differences between Great Lakes colonies (Table 3) may also be ascribed to the action of toxic chemicals including 2,3,7,8-TCDD. The capacity of 2,3,7,8-TCDD to impair the hepatic

accumulation of vitamin A has been demonstrated in rats (Thunberg et al., 1979). Table 3 clearly shows that 2,3,7,8-TCDD exposure is greater and retinoid concentrations are lower in the Lake Ontario gulls compared with gulls from Lakes Superior and Michigan. Significant levels of other highly toxic dioxins were also reported in the 1983 gull eggs and their pattern between lakes was the same as that of 2,3,7,8-TCDD (Stalling et al., 1985). Thus, the possibility exists that exposure to dioxin-like contaminants is at least partially responsible for the lower retinoid concentrations in Lake Ontario gulls.

Of the complex chemical mixture contaminating the Great Lakes ecosystem (Mineau et al., 1984), dioxin-like compounds are particularly effective in altering the activity of liver enzymes. A relatively high level of hepatic AHH activity was determined in herring gull embryos collected from Lake Ontario in 1981, and differences in AHH activity between Great Lakes colonies corresponded with dioxin levels in gull eggs (Ellenton et al., 1985). The significant relationship between AHH activity and liver retinol concentration (Fig. 8) may be interpreted to mean that enzyme activity dependent upon the cytochrome P_{448} monooxygenase system increased the rate of retinol metabolism. The conversion of retinoic acid to 4-hydroxy retinoic acid and the conversion of 4-oxoretinoic acid to more polar metabolites are thought to involve the monooxygenase system. These reactions occur in the microsomal fraction of various tissues, require NADPH and oxygen, and they are inhibited by carbon monoxide (Roberts et al., 1980). However, increased retinoid metabolism did not result after administration of

the cytochrome P_{448} inducer, 3-methylcholanthrene (Roberts et al., 1980), which means that the precise relationship between the monooxygenase system and decreased hepatic retinoids has yet to be defined. Increased activity of the enzyme UDP-GT has been implicated in decreased retinoid storage in rats dosed with 2,3,7,8-TCDD (Thunberg and Ahlborg, 1980). When ring doves were dosed with the dioxin analogue, however, no relationship was apparent between liver retinoids and UDP-GT activity.

The fact that retinol, and not retinyl palmitate, was affected by the dioxin analogue indicates preferential metabolism of this retinoid. A similar phenomenon may have occurred in the gulls; that is, the ratios of average retinol-to-retinyl palmitate concentrations are 0.77, 0,68, and 0.57 for Lakes Michigan, Superior, and Ontario, respectively. This decreasing ratio of non-stored:stored retinoids was evident despite the lower retinyl palmitate concentration in Lake Ontario gulls.

The results of this study plus that of Moccia et al. (1985) indicate the possibility that environmental contaminants may have affected the thyroid and vitamin A status of herring gulls. In the case of hepatic retinoids, injections of a dioxin analogue duplicated the apparent in-field effects. Could the gull thyroid anomalies also be duplicated in controlled experiments?

CHAPTER 4

INFLUENCE OF NUTRITIONAL LIMITATIONS OF IODINE AND VITAMIN A IN THE RING DOVE AND POULTRY CHICK

Experimental diets may be used to probe mechanisms. In the following studies, dietary concentrations of iodine and vitamin A have been used to stress the thyroid and vitamin A regulatory systems. On the basis of previous research with rodents, it was hypothesized that dioxin-like compounds affect the avian thyroid in a manner comparable to that of low dietary iodine. The combined effect of low iodine plus 3,4,3',4'-TCBP was expected to be synergistic with respect to decreased serum thyroid hormones, thyroid histology, and the development of hypothyroidism. Also, iodine insufficiency was used because it is relevant to possible iodine limitations in Great Lakes wildlife. The results of retinoid analyses in herring gull livers (Chapter 3) point to the possibility of decreased vitamin A intake in Lake Ontario gulls, and/or the reduction of vitamin A stores by environmental contaminants. Again, as a mechanistic tool and to simulate interactions in the environment, low vitamin A and low iodine-low vitamin A diets were utilized.

4.1. Low iodine studies with ring doves

4.1.1 Rationale and experimental design

In histopathologic examinations of fish populations in the Great Lakes of North America, a relatively high incidence of thyroid anomalies have been detected (Black and Simpson, 1974;

Drongowski et al., 1975; Sonstegard and Leatherland, 1976). Recent investigations have revealed similar thyroid anomalies in herring gulls, Larus argentatus, sampled from the Great Lakes (Moccia et al., 1985). The most pronounced anomaly is hyperplastic goiter comprising the enlargement of the follicle epithelia and reduction of the colloid. The occurrence of this goiter-like condition is tentatively linked to environmental contamination by organochlorine chemicals. When fish were collected from the Great Lakes and incorporated into diets fed to fish and rats, thyroid effects were elicited (Leatherland and Sonstegard, 1980, 1982; Villeneuve et al., 1981; Hilton et al., 1983).

Upon closer inspection, the thyroid effects elicited during laboratory toxicity studies with fish do not include the histologic profile observed in Great Lakes fish. That is, fish which received diets containing PCB and mirex (Leatherland and Sonstegard, 1977, 1978, 1979, 1980) or diets containing contaminated Great Lakes fish (Leatherland and Sonstegard, 1982; Hilton et al., 1983) failed to develop hyperplastic goiters. Historically, low iodine has been considered responsible for endemic (hyperplastic) goiters in humans and fish in the Great Lakes region, and the hyperplasia in fish prior to organochlorine contamination could be reversed by the administration of either thyroid preparations or iodine (Marine and Lenhart, 1914; LaRoche, 1952). The lack of a hyperplastic effect in the recent feeding studies (Leatherland and Sonstegard, 1980, 1982; Hilton et al., 1983) may be related to the use of hard, well water and mineral-supplemented diets which would assure a high iodine intake. Moccia et al. (1981) rebut the involvement of

iodine deficiency by noting that sufficient iodine occurs in the Great Lakes to meet the biosynthetic requirements for thyroid hormones. Sonstegard and Leatherland (1976) argue that the incidence of goiters does not correspond with inter-lake differences in iodine levels. Also, the between-lake incidences of hyperplastic goiters in birds (Moccia et al., 1985) parallel those determined in fish (Moccia et al., 1981) indicating that physiological responses of fish involving the pituitary-thyroid axis during the spawning migration may be of minor significance.

The present studies were designed to investigate possible interactions between low iodine intake and the goitrogenic effects of dioxin-like contaminants. Previous laboratory studies have revealed a variety of thyroid disorders in organochlorine exposed birds (Jefferies, 1975) but the interaction with iodine insufficiency has not been previously examined in fish, birds, or mammals.

Doves which had been raised to adult size on the low iodine or normal iodine semi-purified diets were given i.p. injections (0.16 ml) of corn oil or corn oil plus 3,4,3',4'-TCBP (Analabs, New Haven, CT) as a suspension. An acute study was conducted by dosing on day one with 0 or 60 µg/g body weight of 3,4,3',4'-TCBP and sacrificing the birds seven days later by CO_2 inhalation. A 28-day study was conducted by dosing on days 1, 10, and 20 with 0 or 20 µg/g body weight of 3,4,3',4'-TCBP. Metabolic rate was estimated by direct calorimetry on days 21-26 of the 28-day study.

Immediately following sacrifice, a pre-warmed thermistor probe was inserted under the keel into the liver-cardiac region and the

maximum temperature recorded. Cardiac blood was collected and the serum stored at -90°C until analysis. The condition of the thyroid glands was assessed by light microscopy.

Using a complete block design, the experiment was conducted with three doves per replicate and two replicates per treatment (i.e. N=6 per treatment). The data were first evaluated by 2-factor (diet X toxicant) analysis of variance in which the significance criterion was $P<0.05$. When variance was significant, specific comparison of means was accomplished by a Dunnett's test (Zar, 1974) at $P<0.05$. No significant deviations from normality were detected in the data following analysis by the Kolmogorov-Smirnov test (Campbell, 1974).

4.1.2 Results and Discussion

Classical histologic investigations in avian species have demonstrated that iodine deficiency causes a hyperplastic appearance of the thyroid gland (Creek et al., 1957). In the acute study, the thyroid follicles from the low iodine doves were mildly hyperplastic with cuboidal or elongated epithelia and small colloid diameters (Fig. 9). This profile is typical of an active avian thyroid (Astier, 1980) and was interpreted to mean that iodine intake was insufficient for the storage of substantial amounts of thyroglobulin colloid. The histologic profile is also consistent with mild hyperplasia due to increased thyroid-stimulating hormone (TSH) activity (Collins and Capen, 1980a, 1980b). The condition of the low iodine doves may be described as euthyroidism because the diet did not alter growth rate (data not shown), core body temperature, serum TT_4, TT_3 or $\%T_3$ uptake (Table 5).

The thyroid glands of doves receiving the low iodine diet and exposed to 3,4,3',4'-TCBP were observed to have large colloid follicles while the epithelial cells were dramatically diminished and in some cases flattened (Fig. 10). The follicle enlargement was statistically significant and explains the significant increase in thyroid weight (Table 5). The histology of the low iodine exposed group is characteristic of the inactive, or slightly active, avian thyroid (Astier, 1980). These histologic changes were accompanied by a small but significant decrease in core body temperature, and significant decreases in serum TT_4 and TT_3 (Table 5). Thus, the low iodine euthyroidism changed to hypothyroidism in the exposed doves presumably owing to decreased conversion of thyroglobulin to

Table 5. Effect of acute, 7-day exposure to 60 μg/g of 3,4,3',4'-tetrachlorobiphenyl on the thyroid status of doves maintained on semi-purified diets containing normal (300 μg/Kg) or low (3μg/Kg) concentrations of iodine.

	Normal iodine diet		Low iodine diet	
	Controls	Exposed	Controls	Exposed
Serum thyroxine (ng/ml)	38.8 ± 1.2[a]	24.6 ± 0.6[b]	32.8 ± 3.4	23.0 ± 2.5[b]
Serum triiodothyronine (ng/ml)	3.05 ± 0.10	2.28 ± 0.09[b]	3.10 ± 0.11	2.23 ± 0.15[b]
Triiodothyronine resin uptake (%)	49.6 ± 1.5	41.6 ± 1.8	49.3 ± 1.7	41.2 ± 5.6
Thyroid weight (mg)	8.6 ± 0.5	9.8 ± 1.3	6.3 ± 0.5	9.6 ± 1.1[c]
Core body temperature (°C)	41.82 ± 0.08	41.54 ± 0.14	41.75 ± 0.08	41.34 ± 0.12[c]
Colloid diameter (μ)	81.0 ± 3.6	79.7 ± 4.2	56.6 ± 3.1	78.3 ± 4.4[c]

a mean ± SE; n=6 doves per treatment group

b significantly less than control fed same diet; Dunnett's t-test; one-tailed; $p<0.05$

c significantly different from control fed same diet; Dunnett's t-test; two-tailed; $p<0.05$

Fig. 9. Thyroid follicles from control doves fed a low iodine, semi-purified diet. The follicles were mildly hyperplastic with cuboidal or slightly elongated epithelia (E) and small colloid (C) diameters. Magnified 500x.

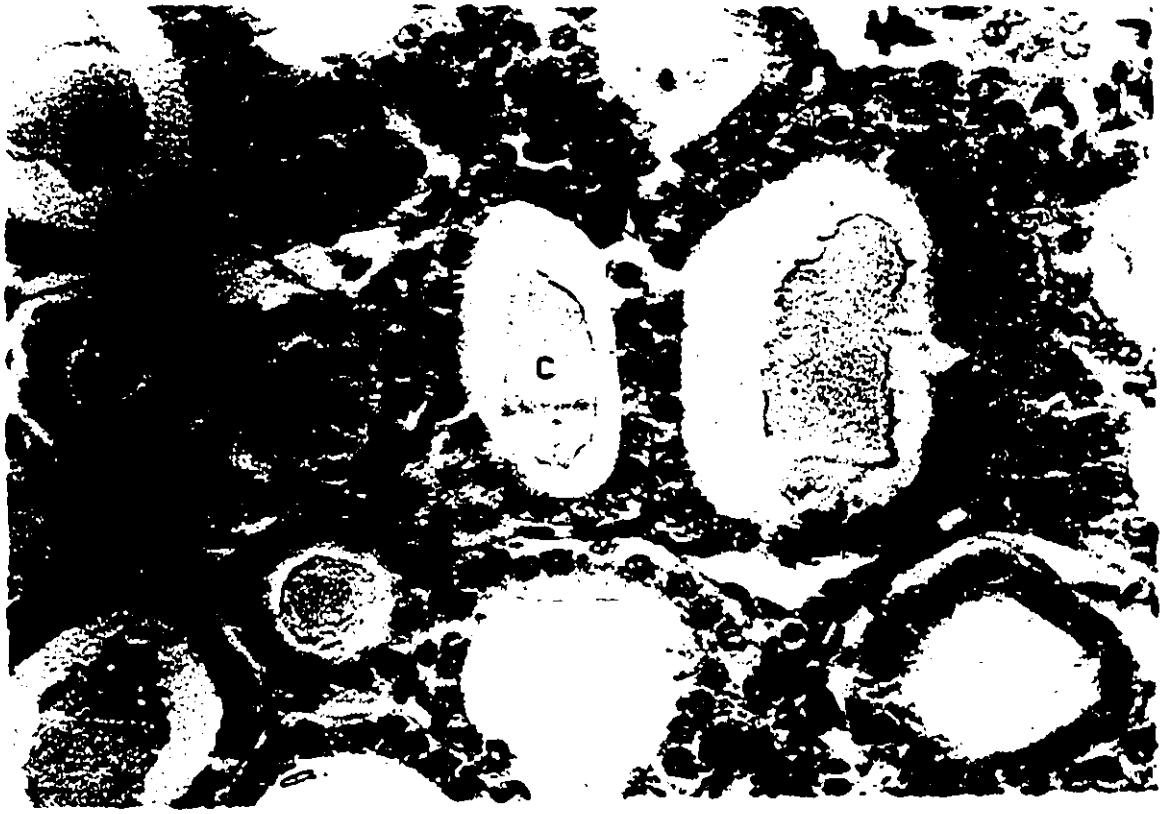


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Fig. 10. Thyroid follicles from exposed doves fed a low iodine, semi-purified diet. Seven days following a single dose of 60 µg/g of 3,4,3'4'-TCBP the epithelia (E) were low cuboidal or flattened and colloid (C) diameters were larger than those of controls (Fig. 9). Magnified 500x.


C

T_4 and T_3. The condition of the exposed doves is consistent with large-colloid goiter as occurs with decreased TSH influence on the thyroid (Turner and Bagnara, 1976).

On the normal iodine diet, the thyroid glands were significantly larger than those of the low iodine controls owing principally to larger colloid diameters (Table 5; Fig. 11). Epithelia were flattened such that the nuclei protruded from the cell surface toward the folliclar centers. Thyroid sections from normal diet controls and normal diet exposed groups were virtually indistinguishable, although serum TT_4 and TT_3 were significantly less in the exposed group (Table 5). Core body temperature showed a tendency to decrease, but the change was not statistically significant.

In the 28-day study, thyroid follicles from both the normal and low iodine diet controls were as described for the acute study. Thyroid weight and colloid diameters were significantly different between the diets, but no effect of the toxicant could be distinguished histologically. The only significant effect associated with 3,4,3',4'-TCBP exposure was decreased serum TT_4 at both dietary levels of iodine (Table 6). Body weight and food consumption were unaffected (data not shown). The fact that 3,4,3',4'-TCBP did not significantly alter core body temperature or metabolic rate (Table 6) indicates that a hypothyroid condition did not develop despite the TT_4 decrease. One may speculate that the doves compensated for decreased TT_4 over the 28-day period, or that toxicant-related oxidative metabolism was thermogenic to the point of balancing the decreased TT_4 activity.

Fig. 11. Thyroid follicles from control doves fed a normal iodine, semi-purified diet were composed of large colloid (C) areas and flattened epithelia (E). Note nuclei protruding towards the follicular center (see arrow). Magnified 500x.

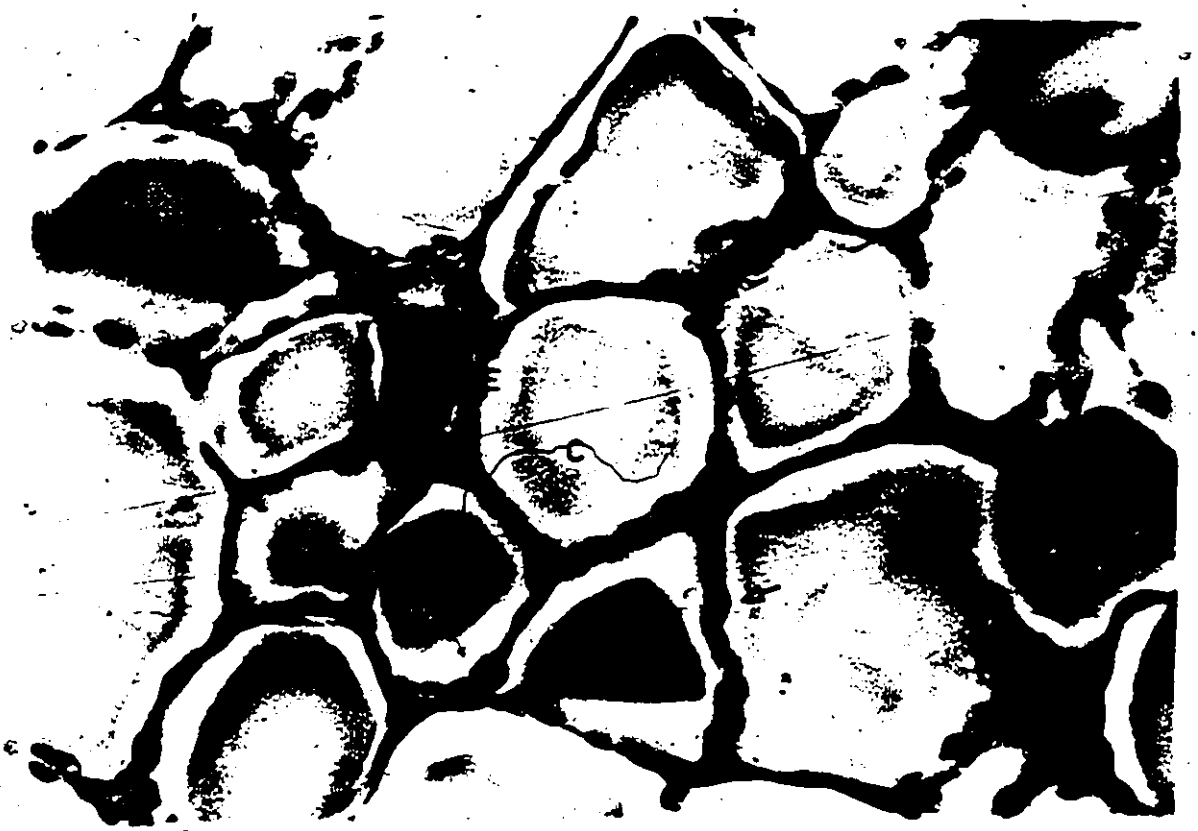


Table 6. Effect of a 28-day exposure to 3,4,3',4'-tetrachlorobiphenyl (3 injections of 20 µg/g) on the thyroid status of doves maintained on semi-purified diets containing normal (300 µg/Kg) or low (3 µg/Kg) concentrations of iodine.

	Normal iodine diet		Low iodine diet	
	Controls	Exposed	Controls	Exposed
Standard metabolic rate (KJ/hr)	4.75 ± 0.05[a]	4.85 ± 0.05	4.83 ± 0.06	4.83 ± 0.09
Serum thyroxine (ng/ml)	36.8 ± 3.2	27.8 ± 2.1[b]	33.4 ± 3.4	25.8 ± 1.7[b]
Serum triiodothyronine (ng/ml)	3.00 ± 0.23	2.69 ± 0.22	3.06 ± 0.10	2.81 ± 0.21
Triiodothyronine resin uptake (%)	48.1 ± 3.5	51.8 ± 1.2	49.0 ± 1.3	48.8 ± 4.2
Thyroid weight (mg)	9.2 ± 0.5	8.3 ± 1.4	6.6 ± 0.7[c]	6.9 ± 0.9[c]
Core body temperature (°C)	41.79 ± 0.09	41.98 ± 0.14	41.73 ± 0.09	41.66 ± 0.88
Colloid diameter (µ)	82.5 ± 4.7	80.3 ± [illegible]	52.7 ± 3.9[c]	58.3 ± 7.0[c]

a mean ± SE; n=6 doves per treatment group

b significantly less than control fed same diet; Dunnett's t-test; one-tailed; $p<0.05$

c significantly different from corresponding normal iodine groups; Dunnett's t-test, two-tailed; $p<0.05$

The present experiments have demonstrated that mild thyroid hyperplasia produced by insufficient iodine was reversed within seven days following acute exposure to the dioxin analogue. The combined action of 3,4,3',4'-TCBP and low iodine upon thyroid histology was therefore antagonistic presumably due to opposite influences upon the TSH feedback system. Extrapolating from these experiments, one may predict this type of antagonism in the case of any organochlorine which potentially causes large-colloid goiter. Conversely, one may predict that hyperplasia caused by any organochlorine would be more severe under conditions of low iodine intake.

A summary of organochlorine effects upon thyroid histology reveals that PCBs caused large-colloid goiters in species closely related to the herring gull (Table 7). Therefore, it is unlikely that PCBs, or the combined influences of PCBs and low iodine, are responsible for thyroid hyperplasia reported in Great Lakes herring gulls by Moccia et al. (1985). Table 7 shows that the literature pertaining to birds is contradictory in the cases of DDT and DDE which have accumulated to relatively high levels in Great Lakes herring gulls (Gilman, et al. 1979; Mineau et al., 1984). The only study conducted using dieldrin in an avian species demonstrated thyroid hyperplasia. Thus, low dietary iodine might be expected to enhance hyperplasia if it were caused by dieldrin. Significant levels of dieldrin have accumulated in herring gulls of the Great Lakes (Gilman, et al. 1979; Mineau et al., 1984).

Organochlorine effects on fish thyroid histology have not been demonstrated in laboratory studies (Table 7).

In the case of mammals, the situation is distinct from that in

Table 7. Comparison of organochlorine chemical effects on thyroid histology between animal classes

Class	Species	Chemical[a]	Thyroid histology	Source
Osteichthyes	Rainbow trout *Salmo gairdneri*	PCBs	No effect	Leatherland and Sonstegard (1979,1980)
	Rainbow trout *Salmo gairdneri*	Mirex	No effect	Leatherland and Sonstegard (1979,1980)
Aves	Rock doves *Columbia livia*	DDT	Hyperplastic	Jefferies and French (1969,1971)
	Quail *Coturnix coturnix*	DDT	Large-colloid	Richert and Prahlad (1972)
	Quail *Coturnix Coturnix*	DDE	Large-colloid	Richert and Prahlad (1972)
	Rock doves *Columbia livia*	DDE	Hyperplastic	Jefferies and French (1972)
	Quail *Coturnix coturnix*	DDA	No effect	Richert and Prahlad (1972)
	Rock doves *Columbia livia*	Dieldrin	Hyperplastic	Jefferies and French (1972)
	Black backed gull *Larus fuscus*	PCBs	Large-colloid	Jefferies and Parslow (1972)
	Guillemots *Uria aalge*	PCBs	Large-colloid	Jefferies and Parslow (1976)

Mammalia	rats	Dieldrin	Hyperplastic	Wassermann et al. (1972)
	rats	PCBs	Hyperplastic	Collins and Capen (1980a)
	rats	PBBs	Hyperplastic	Akoso et al. (1982)
	rats	Photomirex	Hyperplastic	Chu et al. (1981)
	rats	2,3,7,8,-Dioxin	Hyperplastic? (loss of colloid, exfoliated epithelia)	Gupta et al. (1973)

birds. Studies with rats using a variety of organochlorine compounds have consistently resulted in thyroid hyperplasia (Table 7). Accordingly, mammalian species exposed to organochlorine mixtures may develop more severe hyperplasia under conditions of low iodine intake compared with iodine sufficiency. Qualitative differences in response to goitrogens between birds and mammals have been noted by other investigators (Newcomer, 1967; Leung and March, 1975) but the reason for these differences is unknown at present.

4.2 Low iodine - low vitamin A studies with poultry chicks

4.2.1 Rationale and experimental design

The present study was designed to examine some of the possible interactions between low iodine, low vitamin A, and a dioxin-like compound. The poultry chick, Gallus gallus, was specifically chosen as the test organism because it is precoccial like the herring gull chick. Semi-purified diets were utilized as a precaution against naturally-occurring goitrogens which may be present in practical diets.

Chicks were maintained on low vitamin A or low vitamin A-low iodine experimental diets for a 12-day period. On days 2 and 6, they were dosed intramuscularly (i.m.)(0.5 ml) with stripped corn oil or 10μg/g body weight of pure 3,4,3',4'-TCBP dissolved in stripped corn oil. Food consumption was estimated daily by weighing unconsumed food, and body weights were determined every two days. On days 9 and 10, metabolic rates were estimated by direct calorimetry. On day 12, blood was collected from the brachial artery for analysis of thyroid hormones and vitamin A. The birds were sacrificed by decapitation, and the thyroid glands were excised and weighed.

Using a complete block design, the experiment was conducted with 3 chicks per replicate and 2 replicates per treatment. The data were first evaluated by 2-factor (diet x toxicant) analysis of variance in which the significance criterion was $p<0.05$ in a 2-sided F test. Where variance was significant, specific comparison of means was accomplished by a 2-sided Dunnett's test at $p<0.05$. The

sample size was 6 chicks, with the exception of i) liver vitamin A where 4 samples were randomly selected for analysis; and, ii) metabolic rate where sample size is 2 replicates per treatment (3 chicks per replicate tested simultaneously). The influence of the ~~semi~~ purified diets upon thyroid hormone and vitamin A levels was assessed by comparison with practical diet controls (n=3) by 2-sided t-tests at the $p<0.05$ level of significance.

4.2.2. Results

Growth and food consumption

At the start of the 12-day experimental period, the chicks averaged 240g body weight and grew steadily at rates of approximately 10-15 g/day depending upon the treatment. Growth rates on the experimental diets were comparable to published values for chicks receiving practical diets (U.S. NRC, 1977). No overt symptoms of either vitamin A deficiency or iodine deficiency were observed. Control chicks receiving the low vitamin A semi-purified diet grew at a significantly lower rate than control chicks receiving the low vitamin A-low iodine semi-purified diet (Fig. 12). Food consumption was not significantly different between control groups (Fig. 12) and compared favorably with accepted values for practical diets (U.S. NRC, 1977). Exposure to 3,4,3',4'-TCBP resulted in a significant growth rate decrease on the low vitamin A-low iodine diet. Growth was not significantly affected by exposure to 3,4,3',4'-TCBP on the low vitamin A diet. Exposure to 3,4,3',4'-TCBP resulted in a significant food consumption decrease in both groups receiving the experimental diets.

Direct calorimety

Upon transfer to the calorimeter, the chicks demonstrated active exploratory behavior followed by less-active walking and preening. The subdued lighting conditions evoked a gradual quiescence and the birds eventually maintained a resting position with eyes closed. These conditions, achieved within 2 h, are assumed to represent standard metabolism. Figure 13 illustrates that the influence of

Fig. 12. Growth and food consumption of chicks receiving semi-purified experimental diets and injected twice with 10 μg/g of 3,4,3',4'-tetrachlorobiphenyl (Open bars = controls; hatched bars = exposed.) Vertical lines represent SE. * = significantly different from pair-fed group and ** = significant difference between controls at $p<0.05$.

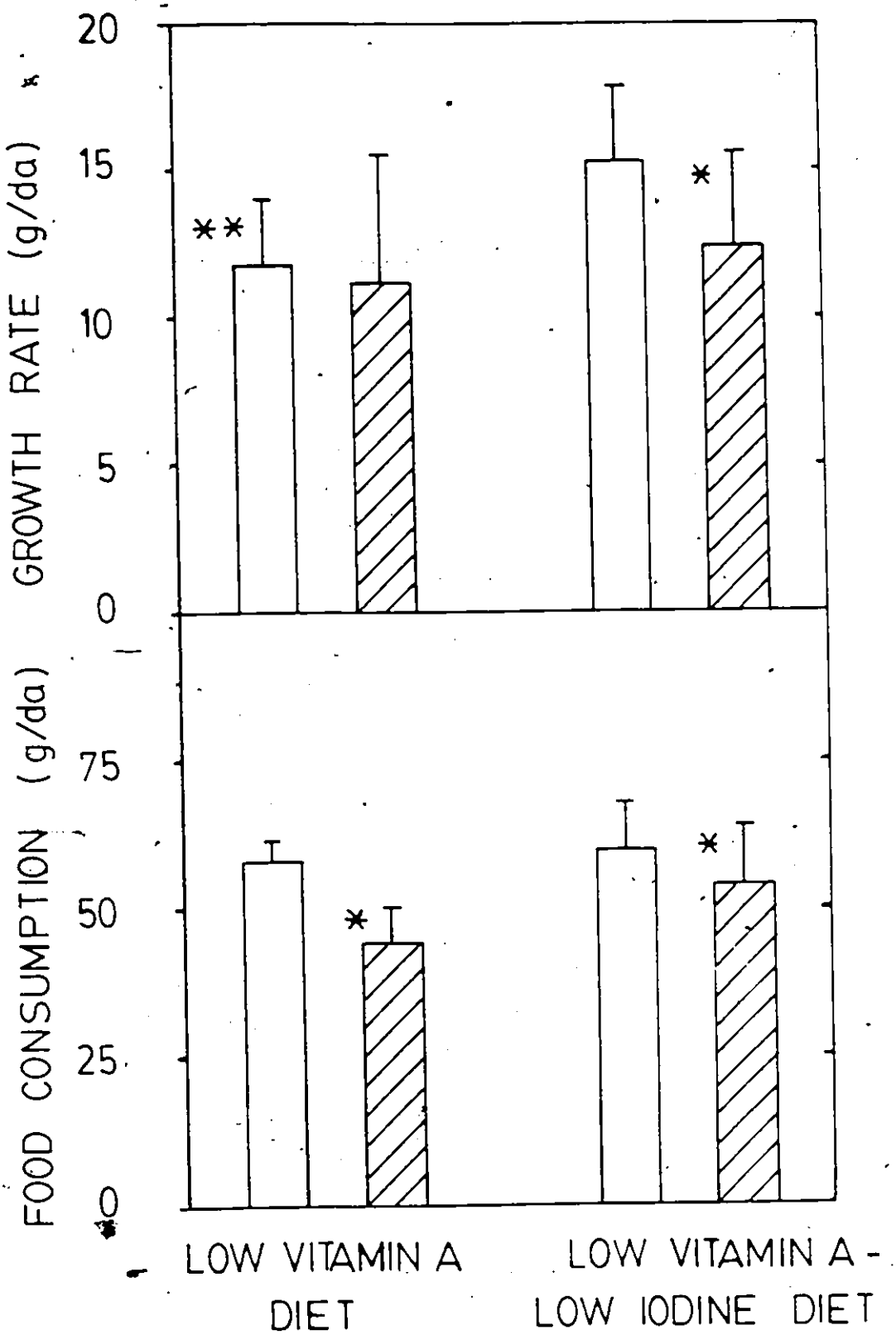

GROWTH RATE (g/da)
20
15
10
5
0
**
*
FOOD CONSUMPTION (g/da)
75
50
25
0
*
*
LOW VITAMIN A DIET
LOW VITAMIN A - LOW IODINE DIET

Fig. 13. Effect of 3, 4, 3', 4'-tetrachlorobiphenyl (2 doses of 10 μg/g) on chick metabolic rates in relation to body size. Each coordinate is the mean value for 3 chicks tested simultaneously in a given trial. Vertical bars represent SE for 5 readings taken during a given test. The standard metabolic rate of nonpasserine birds, Hm, was calculated as $Hm = (Tb-Ta)\ 0.41M^{0.46}$ where Tb is body temperature assumed to be 42^0C; Ta is ambient temperature, 20^0C; and M is body weight. Equation modified from Calder (1974) to give appropriate units.

Low vitamin A - low iodine diet

controls = open circles

exposed = solid circles

Low vitamin A diet

controls = open triangles

exposed = solid triangles

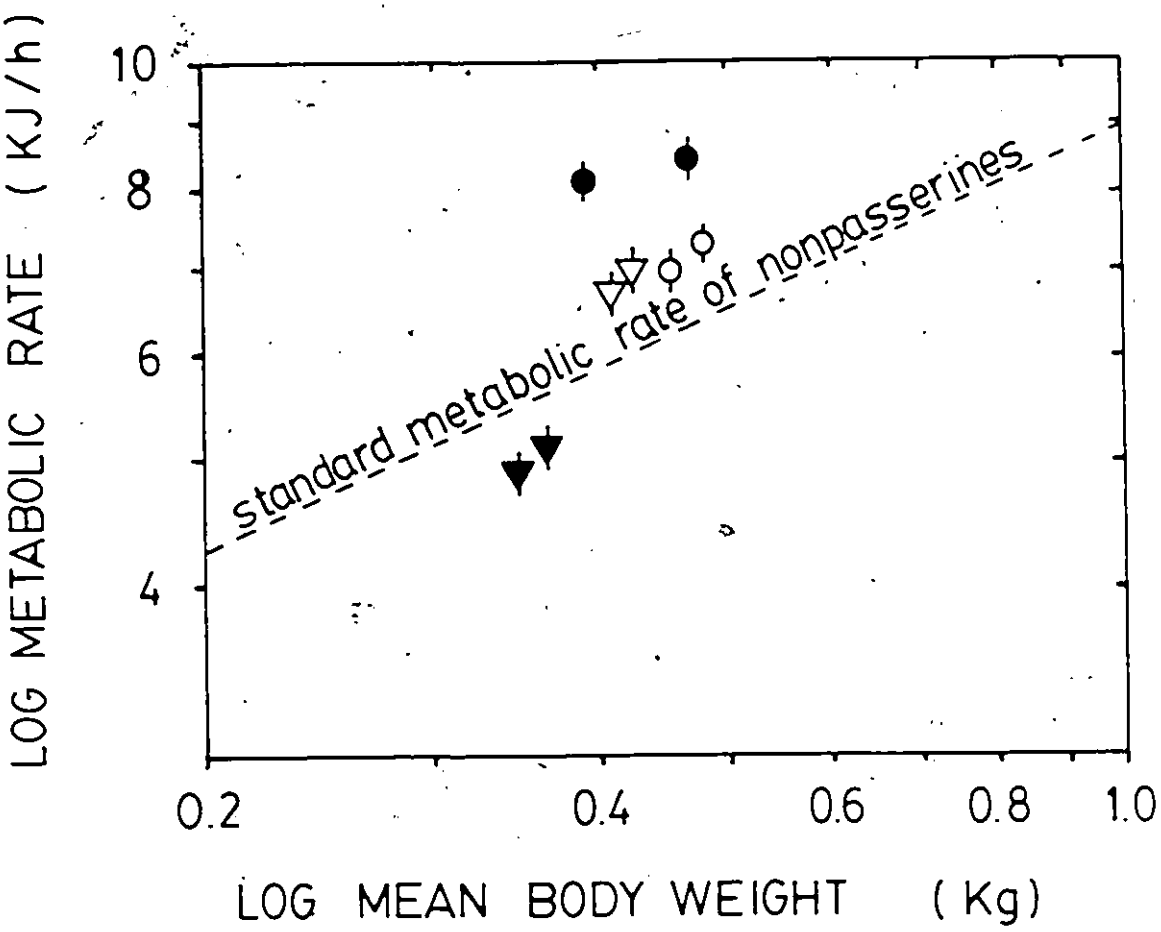

LOG METABOLIC RATE (KJ/h)
10
8
6
4
standard metabolic rate of nonpasserines
0.2
0.4
0.6
0.8
1.0
LOG MEAN BODY WEIGHT (Kg)

TABLE 8. Thyroid status of chicks receiving semi-purified diets and exposed to 3, 4, 3', 4'-tetrachlorobiphenyl (2 injections of 10 µg/g)

		Serum thyroid hormones				Weight of glands
		TT_4 ng/ml	TT_3 ng/ml	T_3 uptake %	FTHI	Mean weight mg/kg body wt
Practical diet	controls (n=3)	18.6±2.1	1.45±0.18	48.0±6.3	12.6±1.8	29.3±2.9
Low vitamin A diet	controls (n=6)	20.3±1.6	1.70±0.18	42.6±3.2	12.2±1.0	28.3±2.4
	exposed (n=6)	10.2±0.7[a]	0.96±0.11[a]	46.6±3.0	6.8±0.6	44.7±3.0[a]
Low vitamin A-low iodine diet	controls (n=6)	19.8±1.5	2.19±0.16[b]	39.5±3.3	11.7±1.6	34.5±1.9
	exposed (n=6)	14.8±2.1	0.91±0.13[a]	57.3±3.8[a]	11.8±1.7	40.8±2.8

TT_4 and TT_3 = total serum thyroxine and total serum triiodothyronine.
FTHI = free thyroid hormone index.

Values are mean±SE; n=number of birds; [a]= significant difference between control and exposed groups receiving the same diet ($p<0.05$, Dunnett's test, 2-sided); [b]= significant difference between controls receiving the practical diet and controls on a semi-purified experimental diet ($p<0.05$, Student's t-test, 2-sided).

TABLE 9. Vitamin A status of chicks receiving semi-purified diets and exposed to 3,4,3',4'-tetrachlorobiphenyl (2 injections of 10 µg/g)

		Serum retinol µg/mL	Hepatic retinol µg/liver
Practical diet	controls	0.51 ± 0.07 (n=3)	———
Low vitamin A diet	controls	0.37 ± 0.02[b] (n=6)	81 ± 23 (n=4)
	exposed	0.31 ± 0.02 (n=6)	44 ± 7 (n=4)
Low vitamin A-low iodine diet	controls	0.43 ± 0.02 (n=6)	82 ± 23 (n=4)
	exposed	0.32 ± 0.03[a] (n=6)	57 ± 21 (n=4)

Values are mean ±SE; n= number of birds; [a]= significant difference between control and exposed groups receiving the same diet ($p<0.05$, Dunnett's test, 2-sided); [b]= significant difference between controls receiving the practical diet and controls on a semi-purified experimental diet ($p<0.05$, Student's t-test, 2-sided).

body size on metabolic rate is minimal compared to the metabolic effect of 3,4,3'4'-TCBP. Significant diet x toxicant interaction occurred; metabolic rates increased in exposed chicks fed the low vitamin A-low iodine diet, but decreased in exposed chicks receiving the low vitamin A diet.

Thyroid status

The results of the thyroid hormone assays are presented in Table 8. With respect to control groups, TT_3 levels were significantly elevated on the low vitamin A-low iodine diet compared with practical diet controls. TT_4 and TT_3 were significantly depressed in exposed chicks receiving the low vitamin A diet commensurate with a significant increase in thyroid weight. Exposed chicks on the low vitamin A-low iodine diet also had lower TT_3 levels compared with controls fed the same diet, but T_3 uptake was significantly greater than that of controls.

Vitamin A status

Table 9 shows that liver retinol levels were not significantly different between any of the treatment groups even though exposed means were 54% and 69% of control values. Placing the chicks on the low vitamin A diet apparently lowered serum retinol levels; low vitamin A control levels were significantly less than serum retinol levels in the chicks fed the practical diet. Exposure to 3,4,3',4'-TCBP resulted in a significant decrease in serum retinol on the low vitamin A-low iodine diet in comparison with controls fed the same diet.

4.2.3 Discussion

Low vitamin A diet: Hypothyroid response to the dioxin analogue

Hypothyroidism typically involves i) decreased blood levels of thyroid hormones, ii) goiter, i.e. alteration of thyroid gland size due to changes in the colloid and/or epithelia sizes, and iii) decreased metabolic rate. Chicks receiving the low vitamin A diet and exposed to the toxicant were clearly hypothyroid in comparison with controls fed the same diet. First, serum TT_4 and TT_3 decreased significantly (Table 8) without a change in the proportion of bound hormone, as indicated by % T_3 uptake. These changes were associated with a 44% decrease in the FTHI (not statistically significant).

Second, in the case of exposed chicks receiving the low vitamin A diet, thyroid weight increased significantly which is indicative of a goitrous condition. Whereas mammals apparently respond to organochlorine poisoning by developing hyperplastic goiters (c.f. Wassermann et al., 1972; Gupta et al., 1973; Collins and Capen, 1980a, 1980b; Chu et al., 1981), PCB exposure in the two avian species investigated thus far resulted in large-colloid goiters (Jefferies and Parslow, 1972, 1976). The essential difference in etiology of the goiters is increased TSH influence in the hyperplastic type as opposed to decreased TSH influence in large-colloid goiters. Thus, large-colloid goiters are associated with decreased release of thyroid hormones. The decreased serum TT_4 and TT_3 levels in exposed chicks, therefore, may be associated with the development of large-colloid goiters.

Third, chicks on the low vitamin A diet not only developed a goitrous condition, but the significant decrease in metabolic rate (Fig. 13) supports the occurrence of hypothyroidism. The decreased food consumption (Fig. 12) may be considered a sign of hypothyroidism, as it is in mammals. However, given the dramatic decreases in circulating hormone levels and metabolic rate, the lack of growth rate reduction is atypical of hypothyroidism and indicates that extra-thyroidal factors may be involved. This point is considered further in relation to serum vitamin A.

Low vitamin A-low iodine diet: Antagonism of the hypothyroid response

Low iodine intake evokes TSH feedback which may initially cause hormone overproduction until the feedback system is further modified by circulating hormone levels. Should the iodine intake remain low, the continued action of TSH will eventually result in a hyperplastic goiter by which serum hormone levels are maintained, i.e. euthyroidism (Mazzaferri, 1974). The iodine content of the low vitamin A-low iodine diet, 70 μg/Kg, was selected as being slightly lower than the threshold for histological changes in the chick thyroid (Creek et al., 1957). The increased TT_3 levels in controls fed the low vitamin A-low iodine diet, as compared with practical diet controls (Table 8), may be attributed to TSH feedback, increased hormone release, and increased conversion of T_4 to T_3 in the blood. Exposure to 3,4,3',4'-TCBP did not elicit a hypothyroid response in chicks receiving the low vitamin A-low iodine diet. The lack of significant effects on TT_4 and thyroid weights (Table 8)

in addition to the increase in metabolic rate (Fig. 13) indicates that the overall effect of the toxicant was quite different between the two semi-purified diets. The decrease in TT_3 levels of exposed chicks may have been counterbalanced by the decrease in binding to serum proteins, as indicated by T_3 uptake (Table 8). Such a situation may exist under certain pathological conditions. For instance, chronic low levels of TT_3 or TT_4 may occur in conjunction with normal thyroid control of metabolism in kidney disease (Recant and Riggs, 1952) - the thyroid status being more closely aligned with free thyroid hormone levels.

As discussed above, PCBs have been demonstrated to elicit large-colloid goiters in birds - an effect related to decreased TSH influence on the thyroid. In contrast, low iodine is known to trigger TSH release causing hyperplastic goiters. As the only intended difference in the composition of the semi-purified diets is iodine content, the effects of 3,4,3',4'-TCBP exposure may have been antagonized by low iodine. Similar results were obtained by March and Poon (1981) for 3-week old chicks treated with thiouracil.

Vitamin A and thyroid hormone transport

Considering the effect of the PCB congener on the low vitamin A-low iodine diet, the significant increase in T_3 uptake (Table 8) may be interpreted to mean a decrease in the concentration of thyroid hormone carrier proteins. Serum T_4 binds with globulin, albumin, and prealbumin (PA) in chicks (Bhat and Cama, 1978a). PA is also the unique carrier protein for vitamin A. That is, stored or newly absorbed retinol in the liver is bound to retinol-binding

protein (RBP) before release into the blood where it is transported as the PA-RBP-retinol complex (Shidogi and Muto, 1977; Bhat and Cama, 1978a). Dietary vitamin A deficiency in rats apparently alters serum levels of thyroid hormones through changes in their transport by PA (Oba and Kimura, 1980; Garcin and Higueret, 1983). Due to the decrease in TT_3 (Table 8) and serum retinol (Table 9) while T_3 uptake increased in exposed chicks (Table 8), the toxicant may have altered serum PA concentrations, or the ability of PA to act as a carrier. A similar mechanism involving decreased PA concentrations was revealed in hyperthyroid (T_4 injected) chicks, although the lack of vitamin A release into the blood resulted in greater liver retinol levels (Bhat and Cama, 1978b). The tendency toward decreased hepatic retinol levels in exposed chicks (Table 9) may involve a separate mechanism, e.g. enzyme activation or induction (Thunberg et al., 1979).

Possible relationship between vitamin A and growth rate

The vitamin A concentration of the diet was 15% of the minimum requirement for chicks fed purified diets (U.S. NRC, 1977) and is, therefore, in a range which would be expected to elicit vitamin A deficiency symptoms. Among the symptoms of vitamin A deficiency in rats is decreased growth rate regardless of whether TT_4 and TT_3 increase (Garcin and Higueret, 1983) or decrease (Oba and Kimura, 1980). Vitamin A deficiency in chicks also causes growth retardation (Barger et al., 1958; Moore, 1957). Plotting growth rate against serum retinol (Fig. 14), a relationship is obtained which supports a correlation between growth and vitamin A under these experimental conditions. A comparable relationship exists for

serum TT_3 levels and growth rate but, as discussed above, $\%T_3$ uptake decreased commensurate with a decrease in TT_3 of exposed chicks receiving the low vitamin A-low iodine diet (Table 8) which indicates that free T_3 levels would not show a consistent relationship with growth rate. Serum TT_4 and FTHI do not exhibit a similar relationship to Figure 14. Thus, it is possible that serum retinol levels influenced growth rate in the present study.

The effect of the toxicant in eliciting hypothyroidism may have been compounded by insufficient vitamin A. Dietary vitamin A deficiency in the rat has produced goiter-like effects (Coplan and Sampson; 1935; Lipsett and Winzler, 1947) in addition to serum thyroid hormone increases (Garcin and Higueret, 1983; Morley et al., 1978) and decreases (Oba and Kimura, 1980). Morley et al. (1978) postulated that such effects may be related to an abnormality in the TSH feedback system, an idea corroborated by the decreased synthesis of thyroid hormones measured by Oba and Kimura (1980). Furthermore, the results of Oba and Kimura (1980) with a vitamin A-deficient diet parallel the PCB congener effects on the low vitamin A diet, i.e. decreased TT_4 and enlarged thyroids (Table 8). Therefore, an inadequate vitamin A intake may predispose organisms to toxicant-related hypothyroidism.

In regard to the tendency for a reduced growth rate in the low vitamin A control groups, as compared with the low vitamin A-low iodine controls (Fig. 14), an interaction between the two dietary factors may be involved. It is interesting that Creek et al. (1957; their Table 1) also found slightly less growth of chicks at 400 µg/Kg dietary iodine than at 100µg/Kg iodine at their lowest level

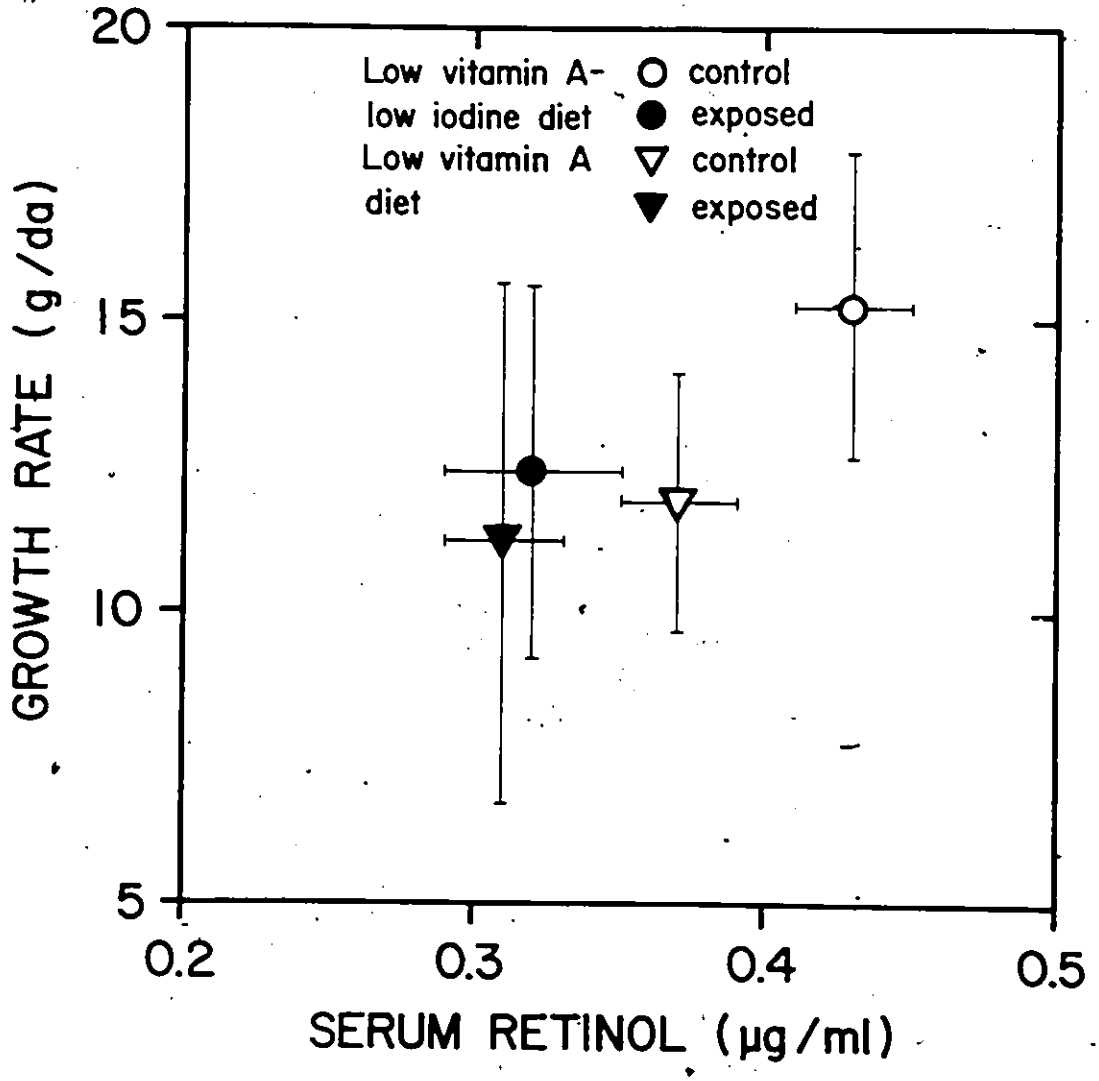
of vitamin A (1200 I.U./Kg), although the data are not statistically significant. The interaction may be related to circulating levels of retinol, as might be interpreted from Fig. 14.

Again, a deficiency of vitamin A in the serum would explain the apparently anomalous lack of growth inhibition in the hypothyroid chicks, i.e. exposed chicks receiving the low vitamin A diet. As discussed earlier, growth inhibition would have been anticipated if hypothyroidism, per se, were involved.

Finally, the increased metabolic rates of exposed chicks on the low vitamin A-low iodine diet (Fig. 13) may be related to serum retinol deficiency. Nutritional vitamin A deficiency in rats elicited a significant increase in metabolic rate (Blaizot and Bénac, 1955); however, an interaction between low vitamin A and increased thyroid hormones cannot be excluded.

In the preceding experiments, a single dose of 60 μg/g of 3,4,3',4'-TCBP in doves fed a low-iodine diet elicited mild hypothyroidism. The same dose in doves fed a normal iodine diet decreased the levels of circulating thyroid hormones. Double doses of 10 μg/g also caused hypothyroidism in poultry chicks receiving a low vitamin A diet. Serum retinol and growth rate decreases occurred in chicks fed a low vitamin A-low iodine diet. Obviously these particular nutritional stressors have profound qualitative and quantitative influences upon the toxicity of dioxin-like compounds in avian species. Especially in the case of growth inhibition, such influences are consistent with the direct involvement of primary toxic mechanisms as stated in the general hypothesis (Chapter 1).

Fig. 14. Relationship between serum retinol and growth rate of chicks fed experimental diets and exposed to 3,4,3',4'-tetrachlorobiphenyl. Vertical and horizontal bars represent SE. Coordinates are obtained from Figure 12 and Table 9.


Low vitamin A-
low iodine diet
Low vitamin A
diet
control
exposed
control
exposed
20
15
10
5
0.2
0.3
0.4
0.5
GROWTH RATE (g/da)
SERUM RETINOL (µg/ml)

CHAPTER 5

CHANGES IN VITAMIN A HOMEOSTASIS AND REPRODUCTIVE INHIBITION IN THE RING DOVE

In the previous chapter, experimental diets were useful in establishing that a dioxin-like compound is capable of causing large colloid goiters and hypothyroidism in avian species. The studies also indicated that decreased serum vitamin A levels might be associated with the manifestation of toxic effects. Despite these valuable mechanistic insights, the use of low iodine and low vitamin A diets may be criticized because an inherent bias is associated with stressing particular physiological systems. The use of experimental diets was therefore abandoned in favour of studies conducted under uniform conditions utilizing full complement nutritional regimens. Various dose levels and exposure times were initially tested to ascertain whether thyroid and vitamin A alterations could be detected and at what doses they occurred. A change in serum thyroxine and/or retinol was expected to correlate with other commonly reported effects of dioxin-like polyhalogenated aromatic hydrocarbons. In the same study, possible connections to hepatic enzyme activities were explored.

5.1 Enzyme studies in juvenile ring doves

5.1.1 Rationale and experimental design

The present study examines the hypothesis that hepatic enzyme induction/activation impairs the regulation of circulating levels of retinol and/or thyroid hormones in the blood. Previous attempts to relate serum retinol concentrations with exposure to halogenated aromatic compounds have yielded inconsistent results (Chapter 1). Extensive studies into nutritional vitamin A deficiency have also demonstrated that serum retinol, per se, is an unreliable index (Underwood, 1974) and that other measurements based on serum retinol provide a better indication of effects, e.g. "relative dose response" (Mobarhan et al., 1981). In the present experiments, therefore, serum retinol concentrations are measured during the absorptive phase of digestion as well as after a 24 h period of food restriction. In addition, a modified version of the "relative dose response" to retinyl palmitate administration is examined. Changes in the various measurements of circulating retinol and thyroid hormones are compared with hepatic enzyme activities and with other commonly reported effects of polyhalogenated aromatic hydrocarbons, i.e. uroporphyrin accumulation in the liver, thymus lymphocyte counts as an indicator of immunosuppression, and enlargement of the spleen and liver.

Ring doves, Streptopelia risoria, were raised to approximately 150 g body weight, i.e. 3-4 months of age, on a standard diet (Pigeon Chow Checkers Ralston Purina Canada, Longueuil, Quebec) supplied ad libitum. The juvenile doves were maintained under controlled aviary conditions of 19-23°C and 14L:10D photoperiod (6:00 to 20:00). The PCB congener 3,4,3',4'-tetrachlorobiphenyl (3,4,3',4'-TCBP; Analabs, New Haven, CT) was administered as single i.p. injections (0.16 ml) suspended in vitamin-stripped corn oil (US Biochemical, Cleveland, OH).

A preliminary study was conducted in which doves received doses of 0 or 20 μg/g body weight. Seven days later, food was restricted at 8:00 AM and blood samples were collected from the brachial artery at various times over a 24-h period.

The principle study involved dosing with 0, 10, 20, or 40 μg/g 3,4,3',4'-TCBP and restricting food for 24 h on days 3, 11, or 51 following the injection. At these time points, "fasted" blood samples were collected from the brachial artery and a gelatin capsule containing 5,000 I.U. retinyl palmitate (cornstarch-gelatin matrix; Sigma Chemical, St. Louis, MO) was placed into the crop by intubation. A second brachial blood sample was collected after 3 h. (Prior experiments had revealed optimum blood retinol concentrations 3 h following retinyl palmitate administration). The doves were returned to an ad libitum food supply for a further 24-h period and were then sacrificed by CO_2 inhalation. Cardiac blood was sampled and the freshly excised right liver lobes were submerged in ice-cold HEPES-KCl (0.02 M HEPES plus 1.15% KCl; pH 7.5; 20% w/v) for microsome preparations. The left liver lobe was

wrapped in polyethylene, quick-frozen in liquid nitrogen, and stored at -90°C for porphyrin analysis. All blood samples were held on ice for 1 h, centrifuged and the serum stored at -90°C. A portion of the thymus was excised for histological observation.

In the preliminary experiment, differences between control and exposed doves were determined by Student's t-test (two-sided; $p < 0.05$). In the second study, the time at which the toxicant was injected, the dose level, and the duration of exposure were randomized. Data were first analysed by the Kolmogorov-Smirnov test for homogeneity of variance and normality. Differences between treatment means (n=4 birds per treatment) were tested by 1-way ANOVA and, if significance ($p<0.05$) occurred, specific comparisons with the controls were subsequently examined by Dunnett's test at the $p<0.05$ level of significance (Zar, 1974). Where appropriate, linear regressions were calculated according to Bartlett's three group method for Model II regressions (Sokal and Rohlf, 1969).

5.1.2 Results

In the preliminary study, doves had high serum retinol levels relative to controls at 7 days following an i.p. dose of 20 μg/g body weight of 3,4,3',4'-TCBP. Food restriction was accompanied by a depression of serum retinol concentrations which were significantly lower than control values 24 h later (Fig. 15).

The results of serum retinol analyses in the second experiment are presented in Table 10. Retinol decreased significantly in 24 h fasted doves at the 20 and 40 μg/g dose levels. When the doves were returned to an ad libitum food supply, serum retinol concentrations in dosed birds showed a tendency to be slightly higher than controls but no significant differences (ANOVA) were detected. Fasted:fed ratios were significantly less than control values at all doses and exposure times with one exception, i.e. the day 12, 10 μg/g treatment group. The relative dose response to retinyl palmitate was not different between treatments (ANOVA).

Liver microsomal AHH activity was significantly greater than controls at all doses and exposure times except the day 4, 10 μg/g treatment group (Fig. 16). AHH activity reached a value 9 times that of controls at the highest dose level 12 days after injection. The activity of liver microsomal UDP-GT was not significantly different between treatments (ANOVA). The highest mean activity for UDP-GT (0.39 ± 0.17 nmol/min/mg protein) occurred 52 days following a dose of 40 μg/g and was 2.6 times that of controls (0.15 ± 0.05). The time-course for the UDP-GT activity change is illustrated in Figure 17.

FIG. 15. Influence of food restriction on serum retinol concentration in control doves (solid line) and doves injected 7 days earlier with 20 μg/g body weight 3,4,3',4'-TCBP (broken line). Vertical lines represent ± SE. Asterisk denotes significant ($p<0.05$) difference from control at identical sampling time.

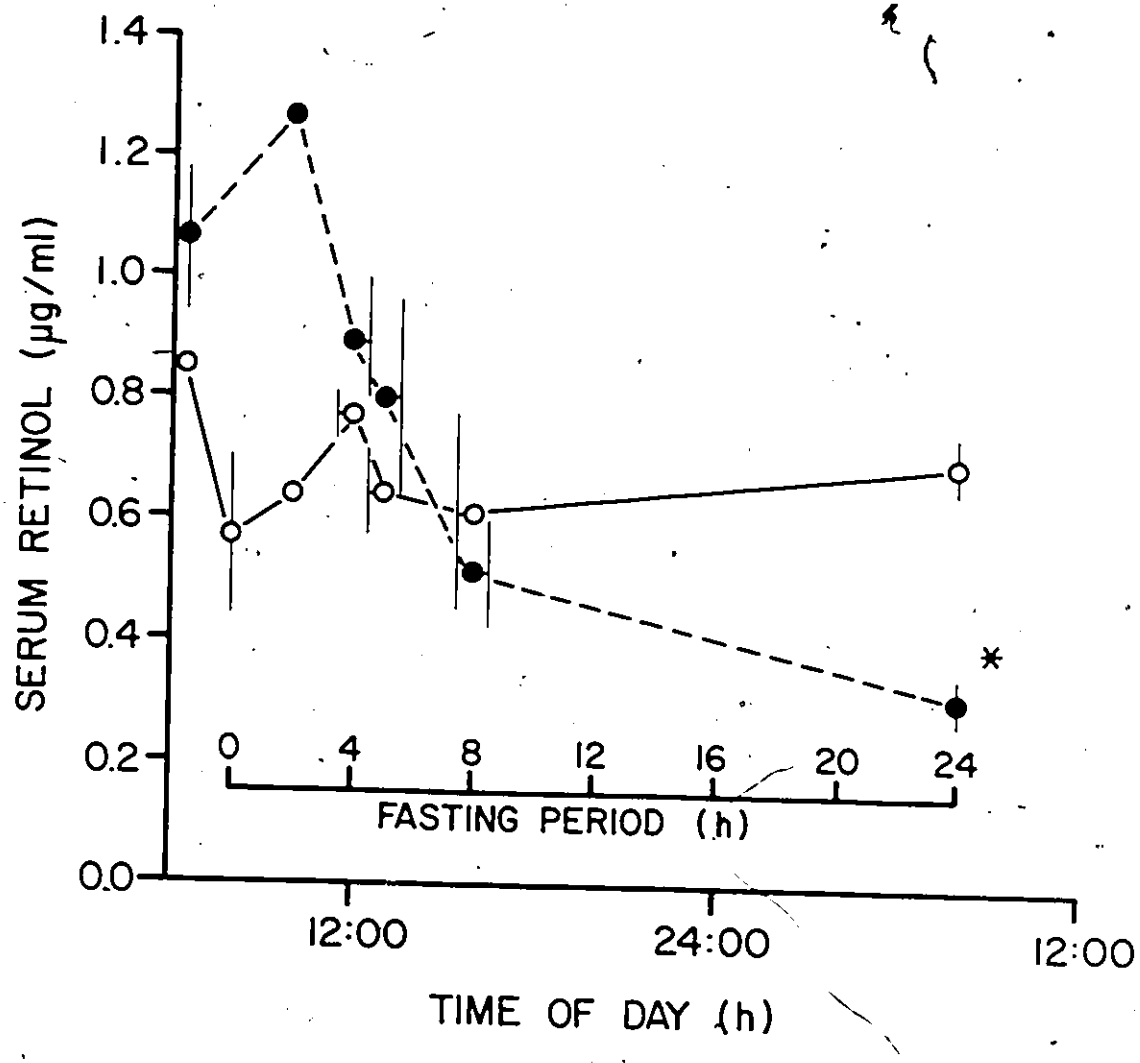

SERUM RETINOL (µg/ml)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
4
8
12
16
20
24
FASTING PERIOD (h)
*
12:00
24:00
12:00
TIME OF DAY (h)

Table 10

Serum Retinol Concentrations in ring doves following single doses of 3,4,3',4'-Tetrachlorobiphenyl[a]

Exposure	Fasted 24 h food deprivation (μg/ml)	Fed No food restriction (μg/ml)	Ratio Fasted: fed	Relative dose response to retinyl palmitate (%)
Control; 12 days	0.70±0.05	0.69±0.04	1.04±0.13	64.6± 4.2
10μg/g; 4 days	0.53±0.03	0.98±0.15	0.62±0.09[b]	54.8± 3.1
10μg/g; 12 days	0.78±0.09	0.87±0.10	0.96±0.22	58.4± 3.1
20μg/g; 12 days	0.32±0.03[b]	0.66±0.08	0.50±0.05[b]	56.5± 6.6
20μg/g; 52 days	0.41±0.01[b]	1.07±0.12	0.39±0.03[b]	50.8± 9.1
40μg/g; 4 days	0.45±0.01[b]	1.18±0.14	0.40±0.05[b]	53.2± 4.8
40 μg/g; 12 days	0.32±0.03[b]	1.02±0.20	0.35±0.08[b]	51.4± 7.1
40μg/g; 52 days	0.48±0.04[b]	1.08±0.11	0.46±0.06[b]	51.2±13.4

[a] Data expressed as $\chi \pm SE$; N=4

[b] Significantly less than control value; Dunnett's test; $p<0.05$.

FIG. 16. AHH activity in liver microsomes as a function of time following single i.p. injections of 10 μg/g (triangles), 20 μg/g (circles), and 40 μg/g (diamonds) of 3,4,3',4'-TCBP. Control values are depicted as inverted triangles. Vertical lines represent ± SE. Asterisks denote significant difference from control at $p<0.05$.

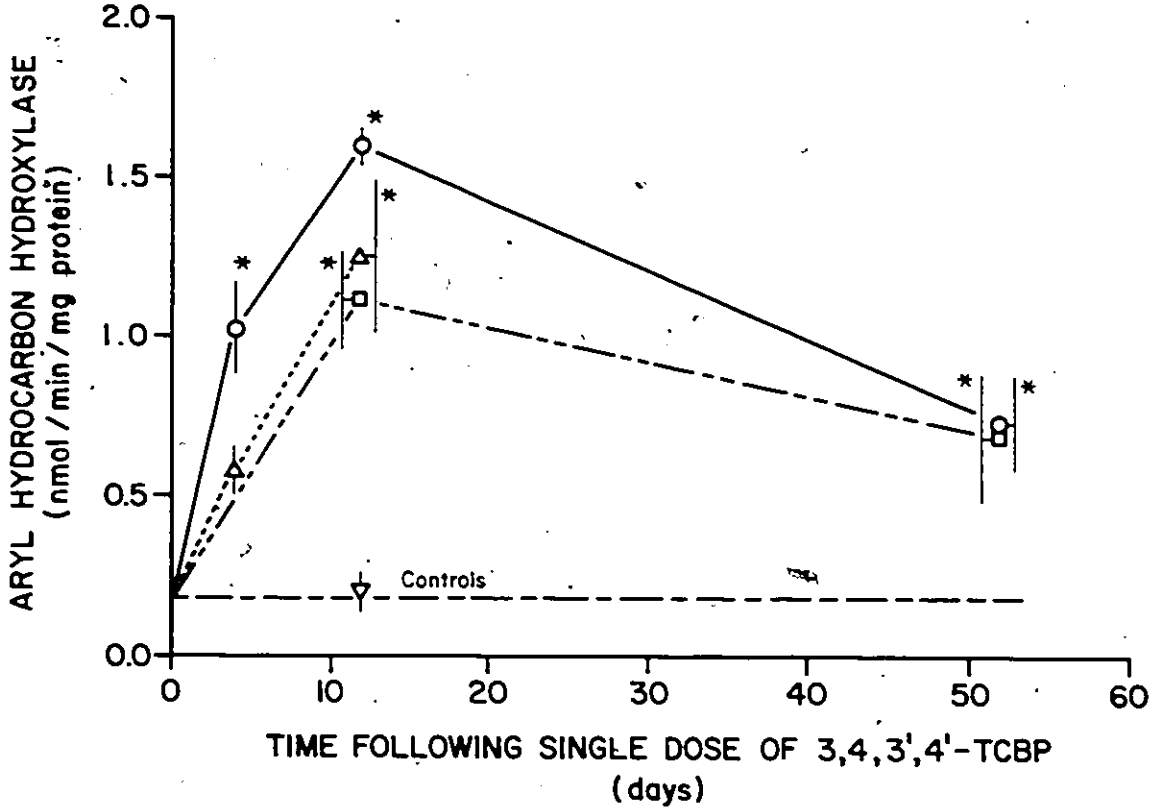
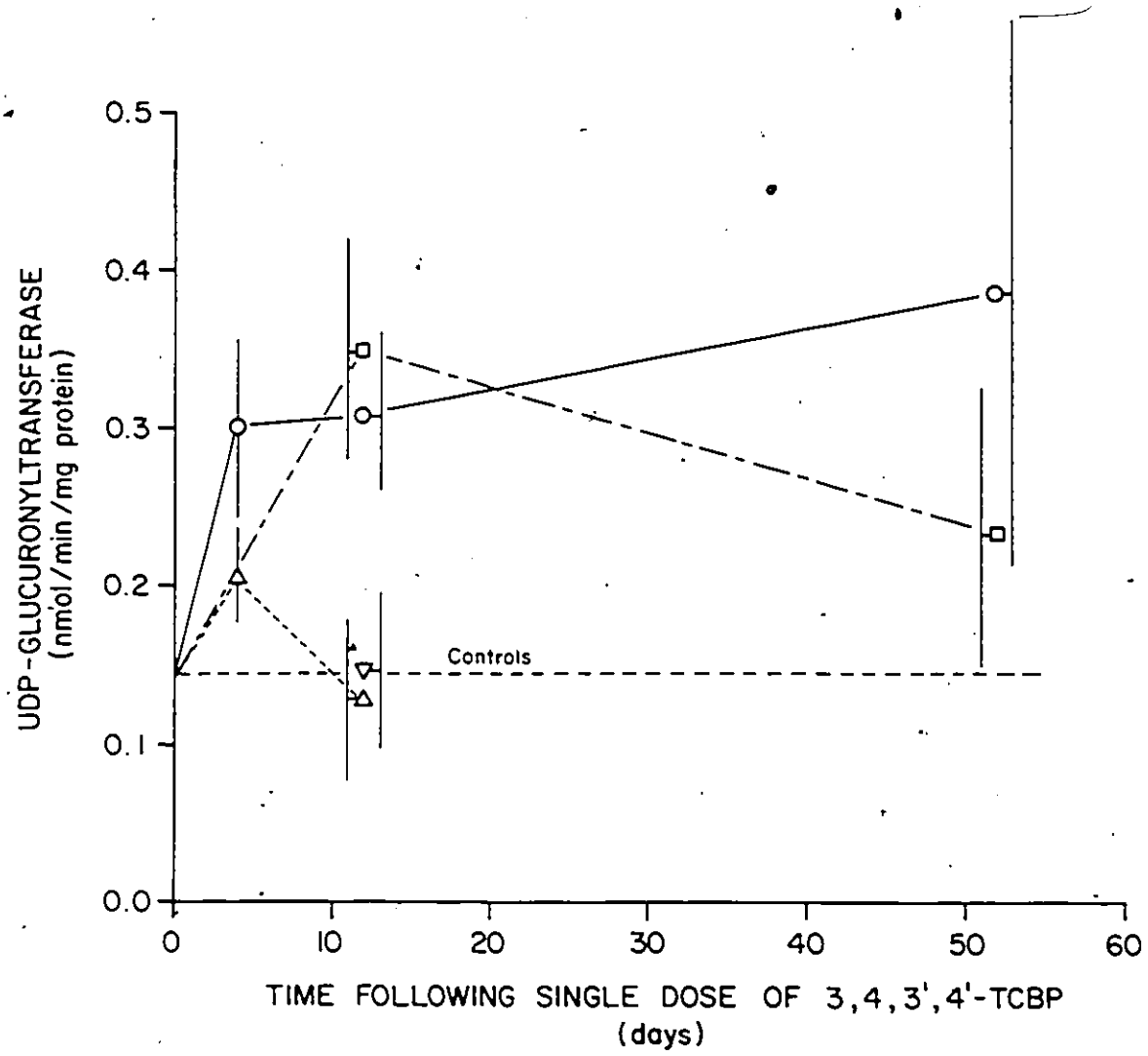

ARYL HYDROCARBON HYDROXYLASE
(nmol / min / mg protein)
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
Controls
TIME FOLLOWING SINGLE DOSE OF 3,4,3',4'-TCBP
(days)

FIG. 17. UDP-GT activity in liver microsomes as a function of time following single i.p. injections of 10 μg/g (triangles), 20 μg/g (circles), and 40 μg/g (diamonds) of 3,4,3',4'-TCBP . Control values are depicted as inverted triangles. Vertical lines represent ± SE.


UDP-GLUCURONYLTRANSFERASE
(nmol/min/mg protein)
0.5
0.4
0.3
0.2
0.1
0.0
Controls
0
10
20
30
40
50
60
TIME FOLLOWING SINGLE DOSE OF 3,4,3',4'-TCBP
(days)

No overt signs of toxicity were observed at any point in the experiments. Body weight (controls = 158 ± 34 g; mean ± SE), liver weight (controls = 3.99 ± 0.34 g) and spleen weight (controls = 54.6 ± 11.3 mg) were not significantly affected by the toxicant although a slight decrease in liver weight and some variation in spleen weight were noted of a dose of 40 μg/g 3,4,3',4'-TCBP. Thymic lymphocyte counts were unaffected (controls = 0.14 ± 0.01 lymphocytes per μ^3). Liver uroporphyrin concentration was not increased by exposure to 40 μg/g (controls = 5 to 10 pmol/g). Thyroid hormones in the serum were unaffected (control TT_4 = 34.5 ± 6.8 ng/ml; control TT_3 = 3.01 ± 0.40 ng/ml; control T_3 uptake = 55.3 ± 0.8%).

5.1.3 Discussion

The preliminary study reveals impairment of serum retinol homeostasis following exposure to 3,4,3',4'-TCBP. The experiment shows that exposure to dioxin-like polyhalogenated compounds may either increase or decrease circulating levels of retinol, and that the direction of this change depends in part upon the digestive phase of the organism. Vitamin A is absorbed from the duodenum (Goodman and Blaner, 1984) which ~~in~~ the doves was cleared of digestible material 5 h after food restriction (data not shown). This time point marks the transition between absorptive and post-absorptive phases and corresponds with the cross-over from higher to lower serum retinol levels in the exposed doves (Fig. 15). The dichotomous serum retinol response to 3,4,3',4'-TCBP serves as a possible explanation for inconsistent results in which serum levels increased, decreased, or did not significantly change upon exposure to different polyhalogenated aromatic compounds (McConnell et al., 1978; Thunberg et al., 1979; Bernert et al., 1983; Darjono et al., 1983; Thunberg and Hakansson, 1983). The influence of digestive phase upon serum retinol levels was generally considered to be negligible, and this notion was supported by postprandial blood sampling in humans (Mejia and Arroyave, 1983). In contrast, the present study demonstrates that the time of blood sampling relative to feeding can have a profound influence upon the outcome of serum retinol analysis where environmental contaminants are concerned.

The decreased concentrations of serum retinol after 24 h of food restriction are of interest in light of the similarity between

symptoms associated with nutritional vitamin A deficiency and those characteristically elicited by dioxin-like polyhalogenated compounds. The ~~results may mean~~ that the retinol supply to tissues is inadequate during the post-absorptive phase and under fasting conditions. The results concur with previous studies in which the combined influence of low dietary vitamin A plus exposure to polyhalogenated aromatic compounds caused a decrease in serum retinol (Darjono et al., 1983; Chapter 4). The common factor is the limitation of dietary vitamin A.

To examine the hypothesis that enzyme induction/activation may be related to the decreased serum retinol levels after 24 h of food restriction, the data for individual doves were plotted against the activities of UDP-GT and AHH. The regressions for each enzyme on "fasted" serum retinol were found to be significant (Figs. 18, 19). Based on what is known of mammalian systems (Frolik, 1984), the metabolism of liver retinol to biliary metabolites involves the irreversible oxidation of retinoic acid to 4-hydroxy-retinoic acid and further oxidation of this metabolite to 4-oxo-retinoic acid. The specific conditions of these reactions are suggestive of cytochrome P_{450} activity (Roberts et al., 1979, 1980). Retinoic acid is also conjugated by UDP-GT to form retinoyl-β-glucuronide (Dunagin et al., 1964; Swanson et al., 1981). The idea that hepatic retinol is degraded by conjugative and oxidative routes induced or activated by polyhalogenated aromatic hydrocarbons is supported by relationships between the hepatic retinol concentration and UDP-GT activity in 2,3,7,8-TCDD exposed rats (Thunberg et al., 1980) as well as AHH activity in 3,4,3',4'-TCBP exposed ring doves (Chapter 3). The

FIG. 18. Regression of post-absorptive serum retinol, i.e. 24 h fasted, with liver microsomal AHH activity in control (solid circles) and exposed groups (open symbols). The exposed group sampled 12 days after a 10 μg/g dose of 3,4,3',4'-TCBP (open triangles) are not included in the relationship. Coordinates represent data on individual doves with values as indicated in Table 10 and Figure 16.

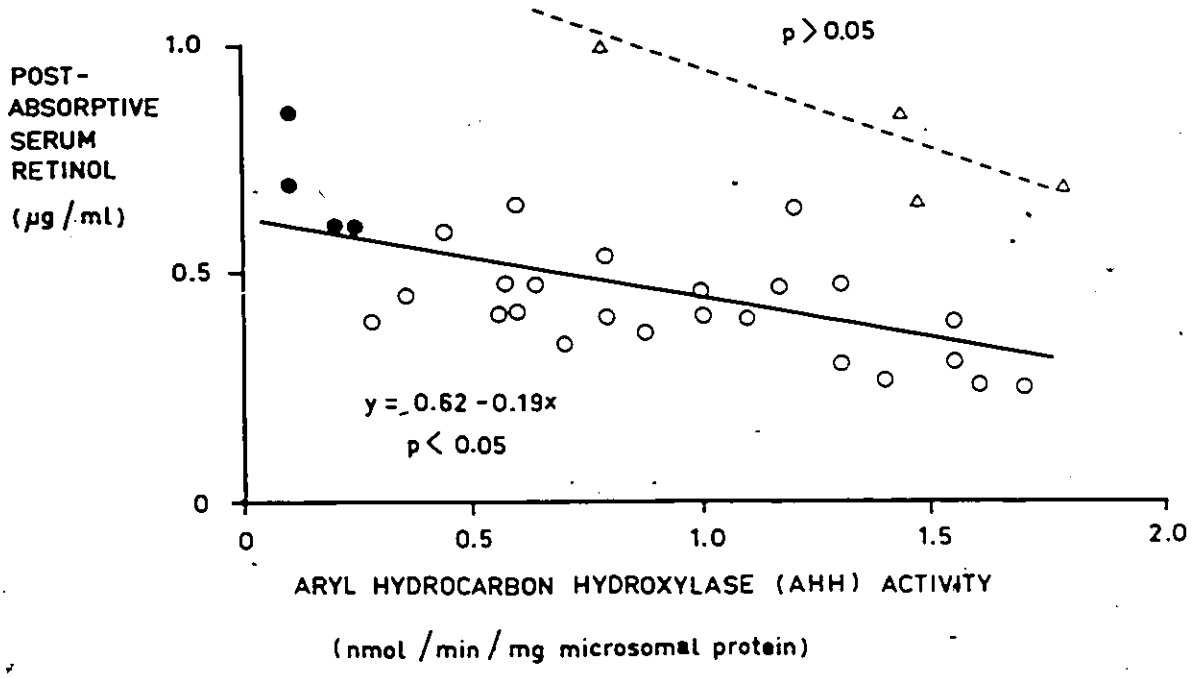
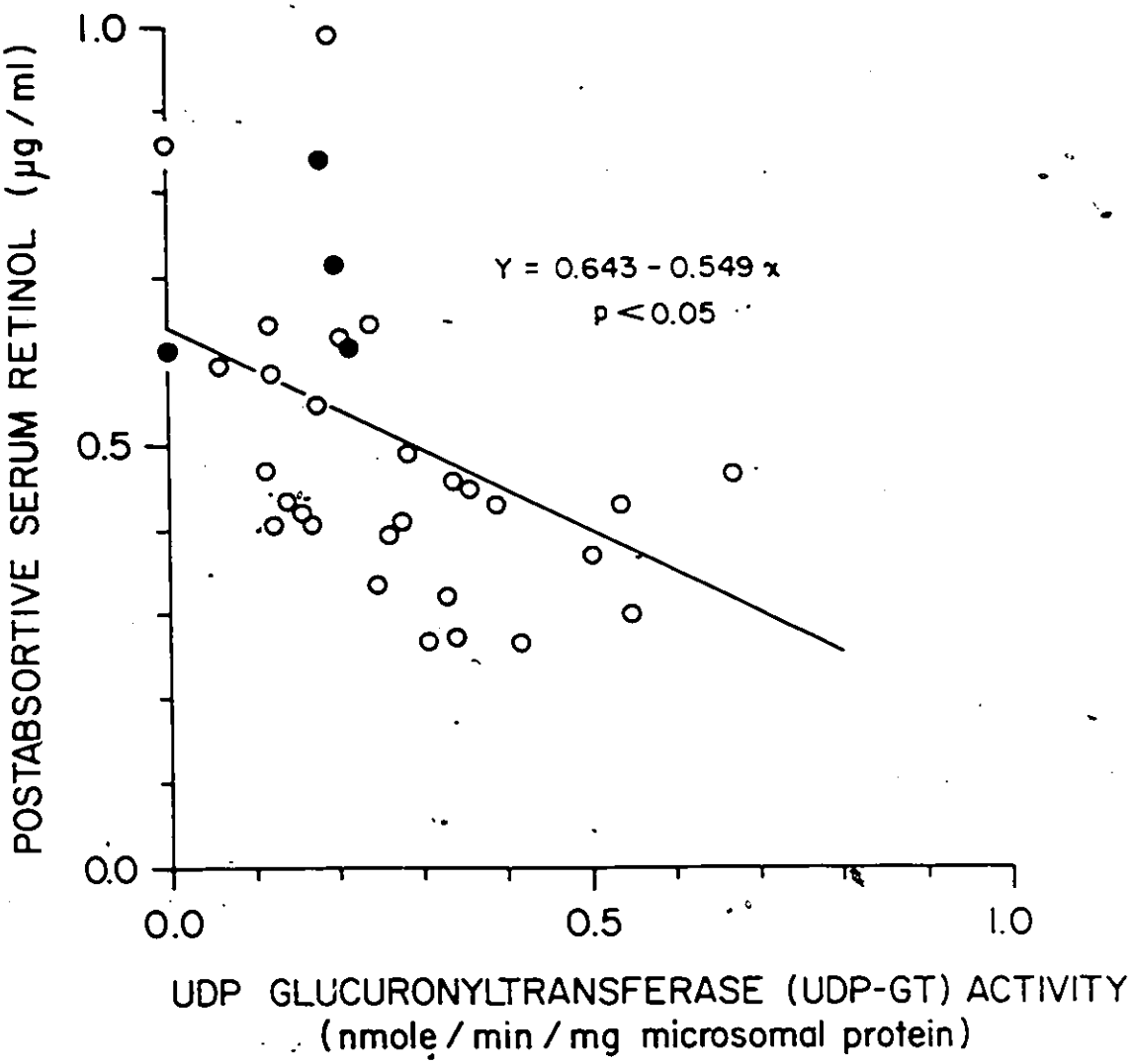

POST-
ABSORPTIVE
SERUM
RETINOL
(μg / ml)
1.0
0.5
0
p > 0.05
y = 0.62 - 0.19x
p < 0.05
0
0.5
1.0
1.5
2.0
ARYL HYDROCARBON HYDROXYLASE (AHH) ACTIVITY
(nmol / min / mg microsomal protein)

Fig. 19. Regression of post-absorptive serum retinol, i.e. 24 h fasted, with liver microsomal UDP-GT activity in control (solid circles) and exposed groups (open circles). Dose levels range from 10-40 µg/g of 3,4,3',4'-TCBP. Coordinates represent data on individual doves as indicated in Table 10 and Figure 17.

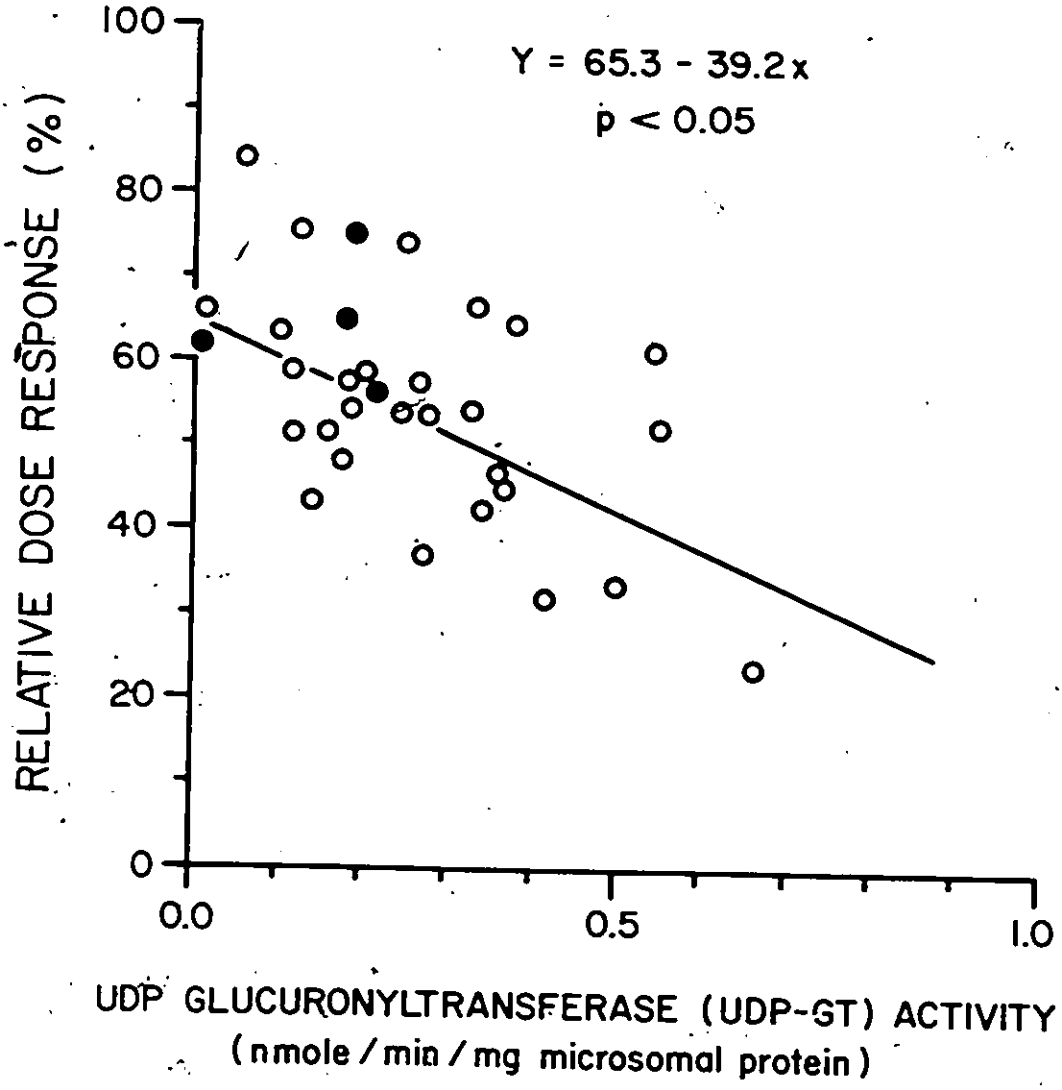

1.0
0.5
0.0
0.0
0.5
1.0
POSTABSORTIVE SERUM RETINOL (µg / ml)
Y = 0.643 - 0.549 x
p < 0.05
UDP GLUCURONYLTRANSFERASE (UDP-GT) ACTIVITY
(nmole / min / mg microsomal protein)

regressions of serum retinol on enzyme activities in the present study may be interpreted to mean that exposure to 3,4,3',4'-TCBP augmented catabolic rates at the expense of circulating retinol. During the post-absorptive phase, enzymatic degradation may have limited the amount of hepatic retinol available for release into the blood. Presumably this was not the case during the absorptive phase because retinol degradation may have been insignificant compared with newly absorbed retinol entering the liver cells.

The results at a dose of 3,4,3',4'-TCBP of 10 μg/g and 12 days exposure are interesting in that coordinates of the serum retinol - AHH regression are distinct from those of the other treatment groups (Fig. 18). The mean value for UDP-GT activity of this group is considerably less than values for the other exposed groups and approximates that of the controls (Fig. 17). Thus, UDP-GT activity may have a predominant influence upon retinol metabolism. When the results of the relative dose response to retinyl palmitate (Table 10) were compared with enzyme activities, no significant relationship was evident for AHH. The regression with UDP-GT was significant (Fig. 20) and may be taken as further evidence supporting a predominant influence of glucuronic acid conjugation upon the retinol imbalance determined under the conditions of this experiment.

Another curious point in comparing the enzymes is the small magnitude of UDP-GT activity relative to that of AHH. Goldstein et al. (1976) reported a similar result in that a dose of 2,3,7,8-tetrachlorodibenzofuran sufficient to increase cytochrome P_{448} in poultry chicks, Gallus sp., failed to activate UDP-GT. These findings are quite different from the mammalian situation in which

Fig. 20. Regression of "relative dose response" with liver microsomal UDP-GT activity in controls (solid circles) and exposed groups (open circles). Dose levels range from 10-40 μg/g 3,4,3',4'-TCBP. Coordinates represent data on individual doves as indicated in Table 10 and Figure 17.


Y = 65.3 - 39.2x
p < 0.05
RELATIVE DOSE RESPONSE (%)
100
80
60
40
20
0
0.0
0.5
1.0
UDP GLUCURONYLTRANSFERASE (UDP-GT) ACTIVITY
(nmole / min / mg microsomal protein)

2,3,7,8-TCDD increased AHH and UDP-GT activities to approximately the same degree (Lucier et al., 1973).

The 3,4,3',4'-TCBP doses used in these experiments were apparently below threshold for several commonly reported effects of polyhalogenated aromatic hydrocarbons. Previous work with avian species has demonstrated effects upon thymic lymphocyte counts, liver porphyrins, and body weights (McKinney et al., 1976; Goldstein et al., 1976), however most of the doses used in these previous studies have been within the lethal range. Even when poultry chicks were exposed to 2,3,7,8-tetrachlorodibenzofuran at a low dose within the lethal range, liver porphyrin accumulation and body weight reduction were not detected (Goldstein et al., 1976). The congener 3,4,3',4'-TCBP is capable of eliciting uroporphyrin and heptocarboxylic porphyrin accumulation in avian liver cells (Zelt, 1980; Swain et al., 1983). With respect to thyroid hormones in ring doves, a single i.p. dose greater than those employed in the present study (i.e. 60 μg/g of 3,4,3',4'-TCBP) resulted in decreased concentrations of both thyroxine and triiodothyronine in serum (Chapter 4).

Whereas a general unifying concept has been developed explaining the molecular action of the highly toxic dioxins, furans, and other related polyhalogenated aromatic hydrocarbons (Nebert, 1979; Poland and Glover, 1980), the connection(s) between liver enzyme induction/activation and the multiplicity of toxic effects has not been conclusively established as yet (Parkinson and Safe, 1981). The present experiments have demonstrated relationships between enzyme activities and decreased serum retinol. Thus, liver enzyme induction/activation may cause dramatic imbalances in the supply of

retinol to extrahepatic tissues with consequent disruptions in cell function. Alternatively or additionally, enzyme induction/activation in extrahepatic tissues might increase retinol degradation. The remarkable similarity between the characteristic effects of polyhalogenated aromatic hydrocarbons and symptoms of nutritional vitamin A deficiency (Vos, 1978; Thunberg et al., 1980; Parkinson and Safe, 1981) means that a primary role of retinoid metabolism in the toxic mechanisms of such compounds is a distinct possibility.

5.2 Reproductive studies with ring doves

5.2.1 Rationale and experimental design

The present study examines the hypothesis that toxicant-related imbalances in vitamin A homeostasis are linked to reproductive impairment. Vitamin A status is assessed by measuring serum retinol in adult doves at the time of mating. Dove eggs are analysed for five retinoid compounds to investigate the possibility that alterations of retinoid metabolism may cause unusual concentrations of various retinoids. Liver porphyrins are analysed because they are widely accepted as a sensitive indication of exposure to polyhalogenated aromatic hydrocarbons.

Ring doves, Streptopelia risoria, were individually housed during a 4-week acclimation period to controlled aviary conditions of 24-25°C, 13-24% relative humidity and 14L:10D photoperiod. Pigeon Chow Checkers (Ralston Purina Canada, Longueuil, Québec), grit plus crushed oyster shells, and water were supplied ad libitum. Controls (9 pairs) received a single 0.16 ml i.p. injection of vitamin stripped corn oil, (U.S. Biochemical Corp., Cleveland, OH), and the exposed group (8 pairs) received a single i.p. injection of 40 μg/g-bw of 3,4,3',4'-TCBP suspended in 0.16 ml of the stripped corn oil. Seven days later, food was restricted for 5 h to minimize variation associated with feeding and a blood sample was collected from the brachial artery. A synchronous reproductive cycle was initiated at this time by reintroducing males into the females' cages and supplying a nesting bowl. All

birds were experienced breeders and the pairs had been tested for compatibility. The doves were observed daily for mating behavior. When egg laying by a given pair was completed, the male was removed along with the nest bowl.

Newly laid eggs were placed in an incubator (Roll-x model, Marsh Co., Garden Grove, CA) at 29-33°C wet bulb and 38°C dry bulb temperatures. Eggs were candled daily and upon signs of pipping were placed in a "hatching chamber" consisting of a microscope slide warmer cushioned with towels at 36-37°C. Hatched chicks were weighed and observed for teratogenic effects. The chicks were further inspected for hydrocephalus and other types of fluid accumulation. Upon dissection, the internal organs were observed, particularly the gonads, and the livers were weighed.

Due to a high incidence of embryotoxicity between days 5 and 7 of incubation during the first reproductive cycle, the experiment was repeated and eggs were sampled to determine possible effects upon retinoid deposition in the yolk and utilization by the embryo. An egg from each clutch was removed from the incubator and frozen on day 3 of incubation after checking for heart beat, and the second egg of each clutch was sampled on day 8 after assessing embryo movement. Daily candling detected embryotoxicity within this time period, and non-viable eggs were immediately sampled. Whole eggs were frozen at -20°C until analysis. Immediately after egg laying, adult male doves were separated from the females and food was restricted for 24 h. Doves were then sacrificed by CO_2 inhalation and the livers were excised, quick frozen in liquid nitrogen and stored at -90°C.

Previous testing had established that serum retinol tended to increase in 3,4,3',4'-TCBP - exposed doves, (Section 5.1). Therefore, the serum retinol data were subjected to the Kolmogorov-Smirnov test of normality and homogeneity of variance with subsequent comparison of means by the Student's t-test (1-sided, p 0.05). Eggs were segregated into 3 groups (control, exposed viable, or exposed non-viable). Within each group, 3 clutches of eggs were randomly selected for retinoid analysis (i.e. 3 eggs/incubation time X 2 incubation times X 3 groups). The resulting data were examined by the Kolmogorov-Smirnov test and the split-plot factorial analysis of variance (Kirk, 1982). According to the outcome of the variance testing, within-group differences attributable to incubation time were independently compared by the paired t-test (2-sided). The results of the first reproductive cycle had revealed that both eggs of a given clutch would either die during incubation or would survive to hatching.

5.2.2 Results

In the first reproductive cycle, all fertile control eggs (n=18 eggs) developed normally to hatching. The median time between pairing and hatching was 8 days and the median incubation time was 16 days.

Clutch size and fertility were not detrimentally affected by the toxicant. A slight delay in laying occurred in the exposed group; the median time to laying was 9 days. Of the eggs laid by exposed doves (n=16 eggs), 31% died at a critical time during early development (i.e. days 5-7 of incubation). A further 12.5% of eggs in the exposed group died during pipping (hatching). All exposed eggs surviving to 1 day post-hatching were comparable to controls with respect to anatomical features (e.g. rudimentary gonads, beak and limb formation) and physiological abilities (e.g. breathing, limb and head movements). With the exception of one breeding pair, both eggs of a given clutch either developed normally or both died during development.

In the second reproductive cycle, the median time to laying was 7 and 9 days for the control and exposed groups, respectively. Embryotoxicity occurred in 37.5% of the exposed eggs potentially developing to the 8-day stage (n=8 eggs sampled on day 3; n=3 arrested; n=5 developing to day 8).

No retinoic acid or retinyl acetate were detected by HPLC in any of the yolks analysed. Consistently low retinaldehyde peaks occurred, but were below the quantifiable limit of detection. All yolk samples contained quantifiable levels of retinol and retinyl palmitate. In control eggs, retinol and retinyl palmitate did not

change significantly during the critical period of development, i.e. days 3 to 8 of incubation (Fig. 21). These retinoids decreased significantly in both the viable and non-viable eggs laid by exposed parents (Figs. 22 and 23). No differences between the exposed and control eggs were identified by analysis of variance.

Serum retinol sampled at the time of mating was significantly greater in exposed male and female doves compared with their respective control groups (Table 11). When data for the exposed females were partitioned according to survival or death of embryos, serum retinol was significantly greater in the females producing viable eggs compared with those producing embryotoxic eggs (Table 11). A comparison of maternal serum retinol and day 3 yolk retinol concentrations is presented in Figure 24 for the control, exposed viable, and exposed non-viable groups.

Among the breeding doves no overt signs of toxicity or behavior anomalies were detected. Uroporphyrin concentrations in adult livers were unaffected by the toxicant, and no differences between sexes were apparent. In all samples, uroporphyrin ranged from 5-10 pmol/g liver. No differences occurred with respect to body weight (controls = 151 ± 2g), thyroid gland weight (controls = 17.1 ± 2.1 mg/pair), liver weight (control males = 1.76 ± 0.03g; control females = 2.17± 0.07g) or kidney weight (control males = 1.02 ± 0.07g; control females = 1.14 ± 0.05g).

Fig. 21. Concentrations as ng/egg of retinol (solid circles) and retinyl palmitate (open circles) in the yolks of control dove eggs measured at two different incubation times. Vertical bars represent SE.

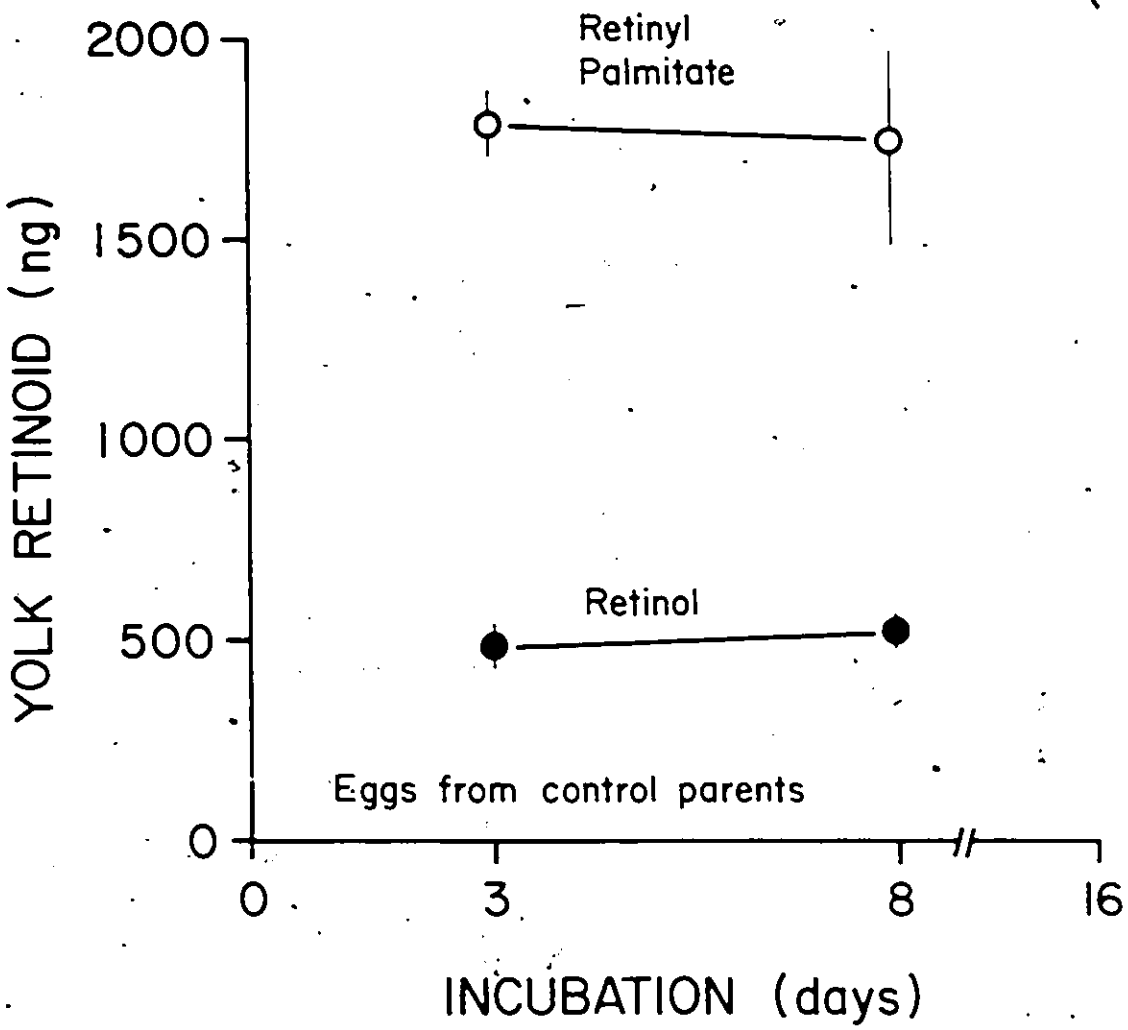

2000
1500
1000
500
0
YOLK RETINOID (ng)
Retinyl
Palmitate
Retinol
Eggs from control parents
0
3
8
16
INCUBATION (days)

Fig. 22. Concentrations of retinol (solid squares; dotted line) and retinyl palmitate (open squares; dotted line) in viable eggs laid by parents which had been exposed to 40 μg/g of 3,4,3',4'-TCBP. Vertical bars represent SE. Mean control values are included (symbols as in Fig. 21).

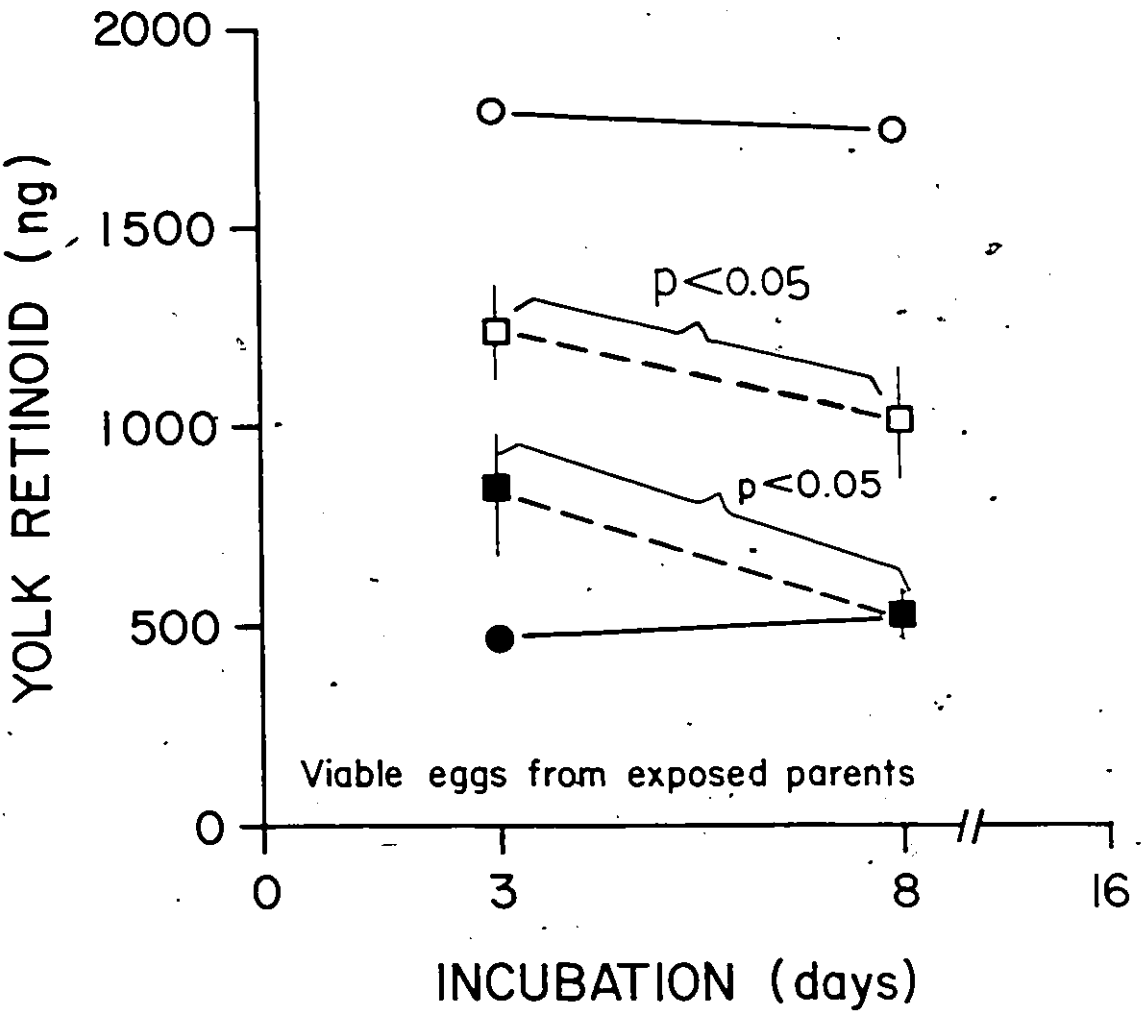

2000
1500
1000
500
0
YOLK RETINOID (ng)
p<0.05
p<0.05
Viable eggs from exposed parents
0
3
8
16
INCUBATION (days)

Fig. 23. Concentrations of retinol (solid triangles; dotted line) and retinyl palmitate (open triangles; dotted line) in egg clutches laid by parents exposed to 40 µg/g of 3,4,3'4'-TCBP in which embryotoxicity occurred prior to day 8 of incubation. Vertical bars represent SE. Mean control values are included (symbols as in Fig. 21).

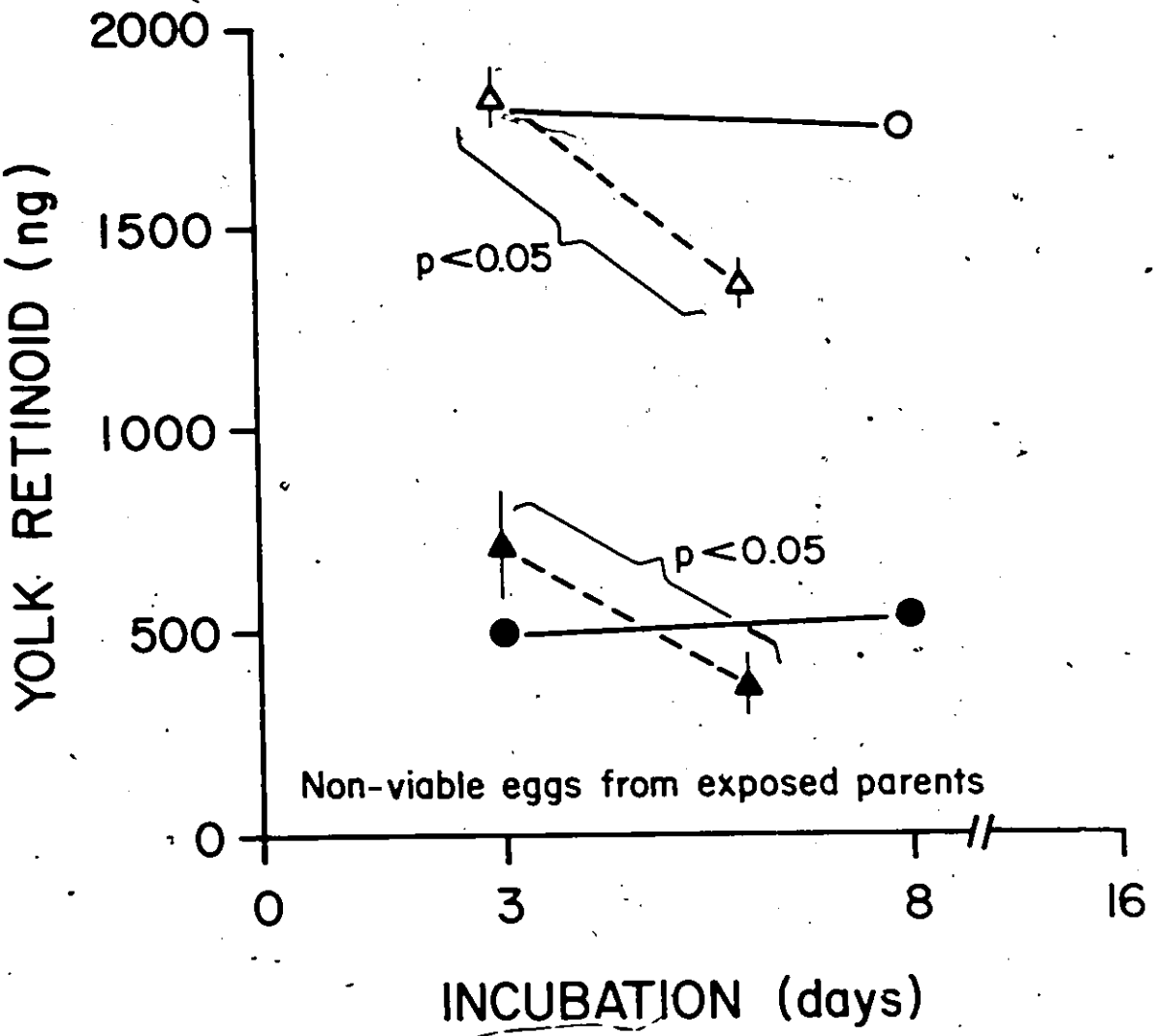

2000
1500
1000
500
0
YOLK RETINOID (ng)
p<0.05
p<0.05
Non-viable eggs from exposed parents
0
3
8
16
INCUBATION (days)

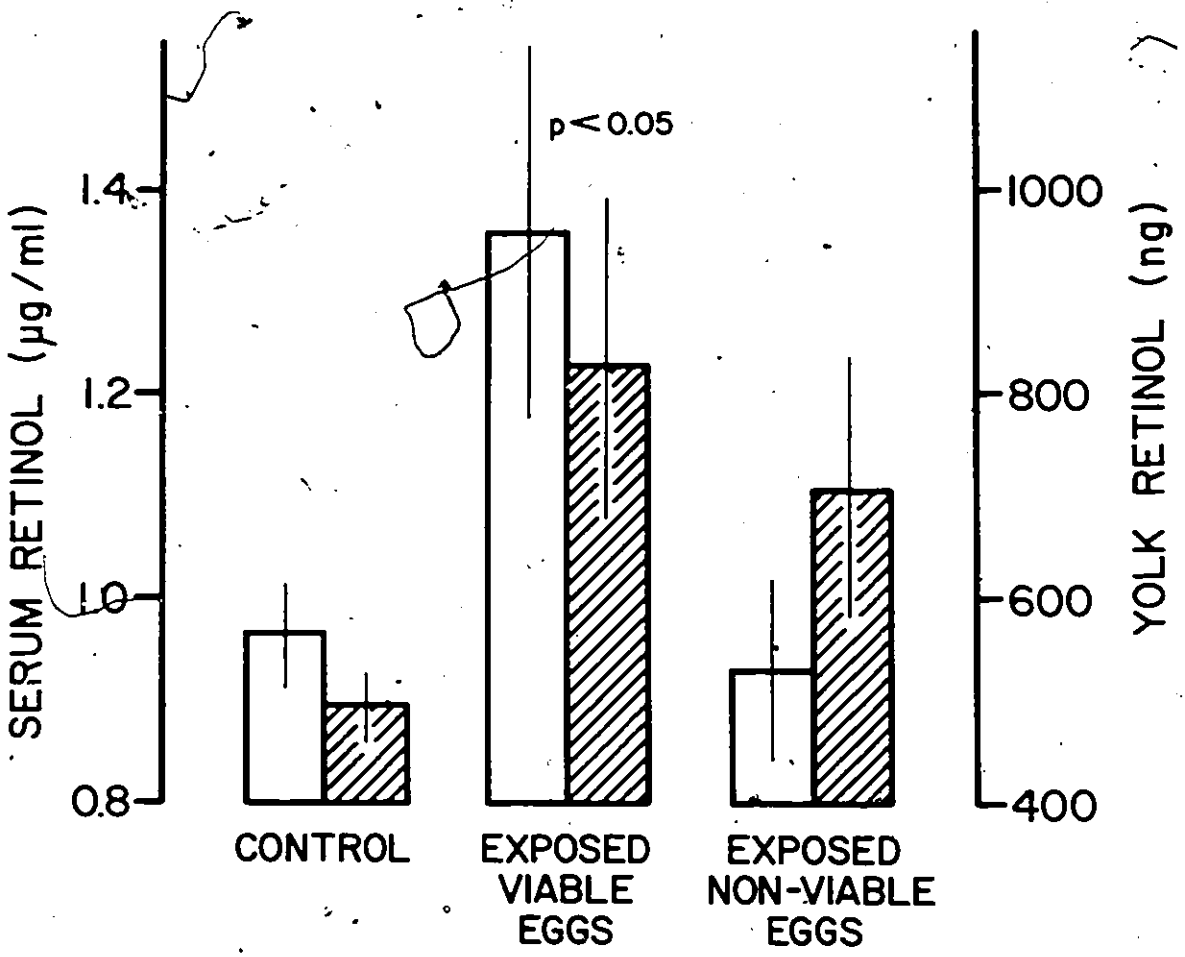
Table 11. Serum retinol (μg/ml) at the time of mating in doves exposed to 40 μg/g of 3,4,3',4'-tetrachlorobiphenyl

	Reproductive cycle		
	#1	#2	#1 plus #2
Males			
control	0.93 ± 0.05 (n=6)	0.87 ± 0.03 (n=7)	0.90 ± 0.03 (n=13)
exposed	1.06 ± 0.09 (n=6)	1.05 ± 0.06 (n=8)	1.06 ± 0.05[a] (n=14)
Females			
control	0.93 ± 0.07 (n=7)	1.01 ± 0.07 (n=8)	0.97 ± 0.05 (n=15)
exposed	1.41 ± 0.27 (n=5)	1.08 ± 0.08 (n=8)	1.20 ± 0.11[a] (n=13)
exposed laying non-viable eggs			0.93 ± 0.09[b] (n=5)
exposed laying viable eggs			1.36 ± 0.10[b] (n=7)

[a] Significantly greater than control ($p<0.05$; one-sided t-test).

[b] Significant difference between "viable" and "non-viable" and groups ($p<0.05$; two-sided t-test).

Fig. 24. Comparison of maternal serum retinol (open bars) and yolk retinol at day 3 of incubation (hatched bars). "Exposed viable eggs" refers to the group in which parents were exposed and embryotoxicity did not occur prior to day 8 of incubation. "Exposed non-viable eggs" refers to the group in which parental exposure resulted in embryotoxicity in one egg of the clutch prior to day 8 of incubation. Vertical lines represent SE. The dose level is 40 ug/g of 3,4,3',4'-TCBP.


SERUM RETINOL (µg/ml)
1.4
1.2
1.0
0.8
p < 0.05
1000
800
600
400
YOLK RETINOL (ng)
CONTROL
EXPOSED VIABLE EGGS
EXPOSED NON-VIABLE EGGS

5.2.3. Discussion

As part of the yolk formation process, greater amounts of retinol are released from the liver and transported to the ovary presumably through the action of estrogens on the synthesis of retinol-binding protein (RBP) (Yeung, 1974). Retinol is deposited in the yolk as a retinol-RBP-prealbumin complex (Sreekrishna and Cama, 1978). Previously reported retinol concentrations in the duck and poultry chicken eggs (Sreekrishna and Cama, 1978; Sivell et al., 1982) are approximately 5-10 times greater than the control levels reported in the present study taking into account differences in egg size. The retinyl ester concentrations reported for ducks (Sreekrishna and Cama, 1978) are much less than the retinyl palmitate control values in ring dove eggs. Retinaldehyde was found to be below the quantifiable limit of detection, i.e. 150 ng/egg, although peaks which coeluted with retinaldehyde standards appeared consistently on the chromatograms. In domestic poultry eggs, retinaldehyde is approximately 16% of the all-trans-retinoic acid concentration (Sivell et al., 1982) which would be 80 ng/egg in a dove-sized egg. Interspecific differences in retinoid deposition in yolk and/or differences in the dietary content of vitamin A may have contributed to the apparent discrepancies between retinoid levels reported previously and those measured in the present study.

The results of this study concur with chronic, low-level exposures of birds to PCB mixtures in which embryotoxicity was found to be a principle reproductive effect (Scott et al., 1971; Cecil et al., 1972; Peakall et al., 1972). Equivalent studies with

the highly toxic dioxins and furans have not been reported. The mode of action of polyhalogenated aromatic hydrocarbons in causing embryotoxicity in mammals and birds is unknown, however chromosome breakages discovered in 3-6 day old ring dove embryos following parental exposure to PCB have been implicated in embryo death at early stages of development (Peakall et al., 1972).

Yolk retinol and retinyl palmitate concentrations decreased significantly in both exposed groups (Figs. 21 and 22) indicating an alteration in the metabolism and/or utilization of these retinoids. Vitamin A is required at early embryonic stages for normal development in birds (Thompson, 1970), and an alteration in the metabolism or utilization of yolk retinoids could affect embryo viability. The fact that decreases in the retinoids occurred in viable as well as non-viable eggs negates the possibility of artifact associated with morbidity. The lowest mean concentration of retinol, 372 ng/egg, was found in the non-viable group, and this raises the question of retinol insufficiency. However, the minimum requirement for normal embryonic development in poultry eggs was determined to be 100-500 ng/egg (Thompson et al., 1969) which converts to 23-113 ng/egg for a dove-sized egg and is considerably lower than the mean retinol concentration in the non-viable group. Also, there is only a slight difference between the control retinol values and that of the non-viable group at death, which further indicates that a simple lack of yolk retinol is not associated with mortality.

Degradative metabolism of retinol in mammals normally proceedes through retinoic acid (Frolik, 1984) which is extremely

toxic to the avian embryo (Thompson et al., 1969; Roberts and Sporn, 1984). Retinoic acid was specifically analysed in the dove eggs to investigate the possibility that this retinoid accumulated in the yolk. Toxic amounts of retinoic acid injected into the yolk sac of domestic chicken eggs are 1-5 µg (Thompson et al., 1969) which would be equivalent to 180-910 ng in a dove-sized egg. Such levels would have been detected in the present study and, therefore, the deposition of toxic retinoic acid concentrations in the yolk did not occur.

The serum retinol response to 3,4,3',4'-TCBP in breeding doves is consistent with that found in juveniles (Section 5.1). Serum retinol distinguished the group of exposed females laying viable eggs from the group laying eggs that did not develop to hatching (Table 11). The significant increase in the serum retinol concentration of exposed females compared with controls was largely due to higher levels in those exposed females which laid viable eggs. Possibly, increased maternal levels of retinol influenced embyro survival and it is interesting to note that the highest retinol levels in day 3 eggs (Fig. 24) occurred in the viable group (i.e. 70% above the control level, but not significantly different from controls). Perhaps greater yolk retinol deposition offset the metabolism/utilization of retinol during the critical period of development.

These experiments clearly show that serum retinol imbalances occur at relatively low doses of a dioxin-like compound, and are detected at dose levels causing embryotoxicity. Several different

measures of serum retinol were quantitatively related to hepatic enzyme activities which raises the possibility of a causal relationship. Changes in yolk retinoid concentrations occurred in eggs laid by exposed parents. No changes in serum thyroxine concentration were evident in the same dose range, i.e. 0-40 μg/g of 3,4,3',4'-TCBP.

CHAPTER 6

CONCLUSIONS

The general hypothesis explored in the present research is that the toxicity of dioxin-like polyhalogenated aromatic hydrocarbons is elicited through alterations in thyroid and/or vitamin A homeostasis. The foregoing experiments utilized several different approaches to examine the general hypothesis, and this was done to specifically test a series of working hypotheses. The previous chapters contain detailed discussions of individual experiments and these will not be reiterated. The intention here is to examine only the salient results and to integrate these parts into a cohesive story.

6.1 Thyroid homeostasis

Low dietary iodine evokes the thyrotropin (TSH) feedback system which increases the activity of the thyroid gland and may thus compensate for the dietary condition. If 3,4,3',4'-TCBP had acted similarly with respect to TSH, then a synergistic effect would have been detected histologically as a further increase in epithelial cell height and decrease in colloid diameter. Unexpectedly, the combined effect of low iodine plus the dioxin analogue was antagonistic in terms of thyroid histology. A single dose of 60 μg/g 3,4,3',4'-TCBP reversed the mild hyperplastic condition of the low iodine doves. In this case, the flattening of follicular epithelia and enlargement of the colloid (i.e. large-

colloid goitre) is not consistent with the effects of PCBs and dioxins in mammals, and is suggestive of a unique avian response to such contaminants. Possibly the dioxin analogue inhibited the release of TSH from the pituitary or inhibited the action of TSH in the follicular epithelia. Decreased TSH activity would lead to decreases in iodine uptake into the gland, lysosomal conversion of thyroglobulin to thyroid hormones, and the release of thyroid hormones into the blood. More thyroglobulin may thus have accumulated in the follicles giving rise to larger colloid diameters. The decreased hormone release may have lead to lower TT_4 and TT_3 levels in serum, and consequent decreases in metabolic rate. This mechanism is distinct from that proposed for mammals by Collins and Capen (1980a, b) which is thought to involve direct interference with thyroglobin conversion. Further evidence supporting a distinct mechanism for birds is the lack of hyperplastic appearance of the thyroid in doves fed a normal iodine diet despite severe thyroid hormone and metabolic decreases at 60 µg/g 3,4,3',4'-TCBP. Also, histologic investigations of PCB-exposed gulls (Jefferies and Parslow, 1972 and 1976) revealed that the avian response was inherently different to that of mammals. The distinction has not previously been recognized in the published literature.

The results of the present studies do not support the hypothesis that dioxin-like compounds per se, or an interaction between dioxin-like compounds and low dietary iodine, are involved in the etiology of hyperplastic-type goiters observed in Great Lake gulls.

Hypothyroidism definitely occurred in doves which had been raised on a low iodine diet and injected once with 60 μg/g of 3,4,3',4-TCBP. Hypothyroidism also developed in poultry chicks receiving a low vitamin A diet and dosed twice with 10 μg/g of 3,4,3',4'-TCBP. Thus, nutritional intake of iodine and vitamin A can influence the development of hypothyroidism. There can be no doubt that serious effects are associated with hypothyroidism, per se, and the possibility exists that higher dose levels or chronic exposures may have revealed a correlation between serum thyroid hormones and serious detrimental effects. The present experiments utilized gross physiological effects such as growth inhibition and reproductive impairment as independent measures of toxicity. At the relatively low dose levels used, growth was not significantly altered in dosed birds that were clearly hypothyroid. A single dose of 40μg/g of 3,4,3',4'-TCBP caused reproductive impairment in ring doves, but no changes in serum thyroid hormone concentrations were evident in adult-sized doves receiving the same dose. Also, the principle reproductive effect, embryotoxicity, is not consistent with known hypothyroid effects. Therefore, the logical conclusion reached under the conditions of the present study is that no evidence was obtained which would support the hypothesis that dioxin-like polyhalogenated aromatic hydrocarbons elicit toxicity through alterations in avian thyroid homeostasis.

6.2. Vitamin A homeostasis

Several effects upon vitamin A homeostasis were demonstrated

in the present studies which corresponded with gross physiological effects:

(i) In poultry chicks fed a low vitamin A-low iodine diet and dosed with 3,4,3';4-TCBP, decreased growth rate corresponded with decreased serum retinol. In the low vitamin A chicks, however, the toxicant did not elicit growth reduction or a decrease in serum retinol.

(ii) At a dose level of 40 μg/g, reproductive impairment occurred in ring doves commensurate with imbalances in serum retinol.

(iii) Significant differences in serum retinol occurred between exposed female doves laying eggs which developed to at least day 8 of incubation, and those whose eggs died before day 8.

(iv) In eggs laid by exposed doves, yolk retinoids decreased significantly between days 3 and 8 of development which is the critical period for embryotoxicity. Comparing control eggs, exposed viable eggs, and exposed eggs which did not develop to day 8, the lowest retinol concentrations were detected in the non-developing eggs.

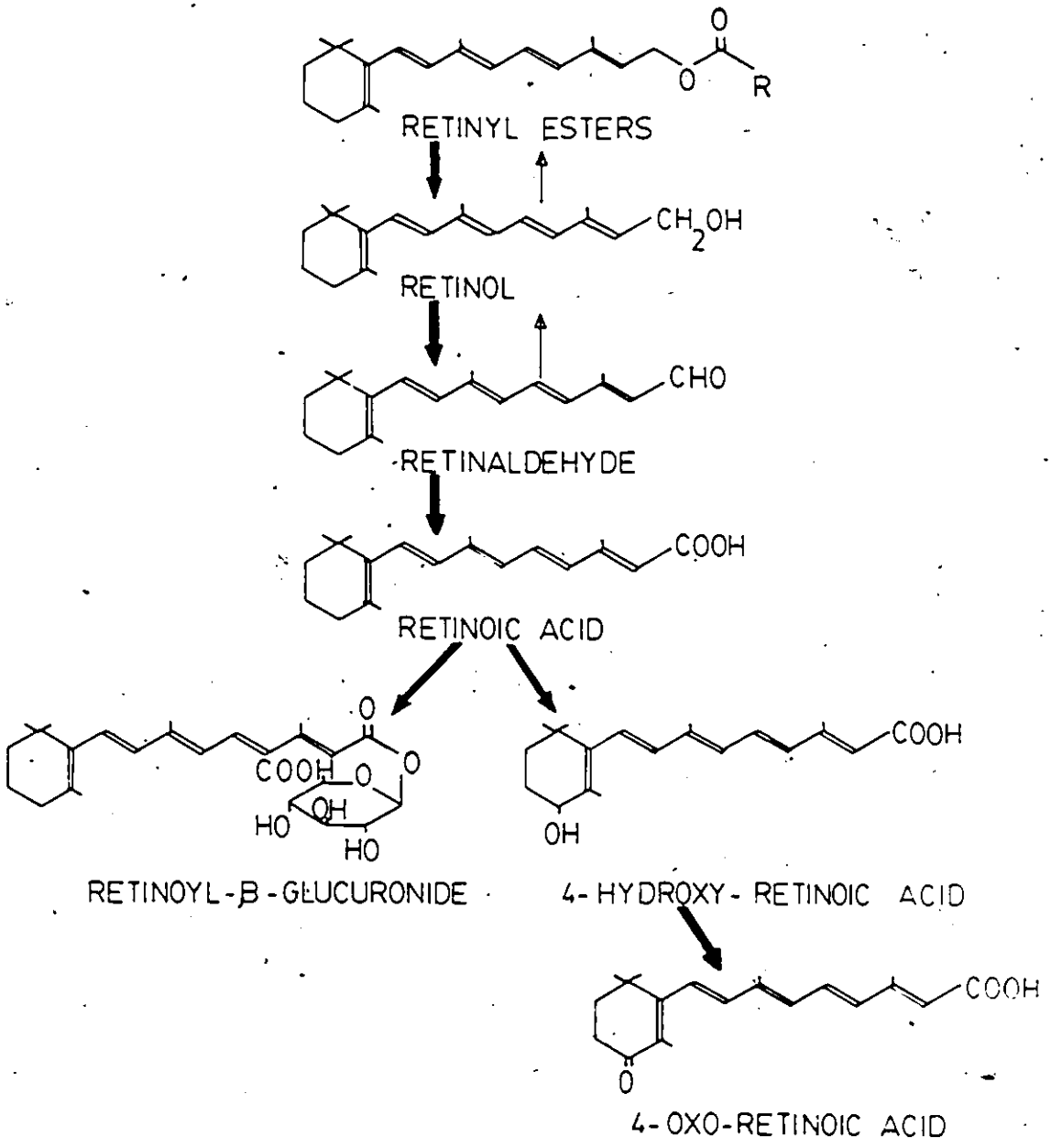
These results support the hypothesis that 3,4,3',4'-TCBP may have elicited toxic effects by altering vitamin A homeostasis under the conditions of these studies.

In the present experiments, a relationship was demonstrated between the induction of liver microsomal AHH activity and the decrease in liver retinol concentration. Such a relationship has not been previously reported in mammals or birds. Furthermore, the present experiments have shown for the first time that the

induction of hepatic AHH and UDP-GT correspond with a decrease in serum retinol (i.e. in 24 h fasted doves injected with 0-40 μg/g of 3,4,3',4'-TCBP). The importance of this connection between the vitamin and enzyme induction is that it could explain the extrahepatic effects of dioxin-like contaminants which are remarkably similar to the effects of vitamin A imbalance. Despite a wealth of evidence produced thus far which implicates AHH in the ultimate mode of action of dioxin-like toxicants, a mechanistic link with actual toxic effects has not been clearly identified. The results of the present research provide a possible mechanism.

Based on what is known of mammalian systems, the pathway for hepatic retinoid metabolism involves the conversion of retinoic acid to 4-hydroxy-retinoic acid which in turn may be oxidized to 4-oxo-retinoic acid (Fig. 25). The induction of AHH may accelerate these reactions. Retinoic acid may also be conjugated to retinoyl -β-glucuronide which is presumably augmented by UDP-GT induction.

Fig. 25. Proposed alterations in the hepatic pathway for retinoid metabolism. Large arrows indicate increased flux due to the action of dioxin-like compounds in inducing enzymes associated with this pathway.

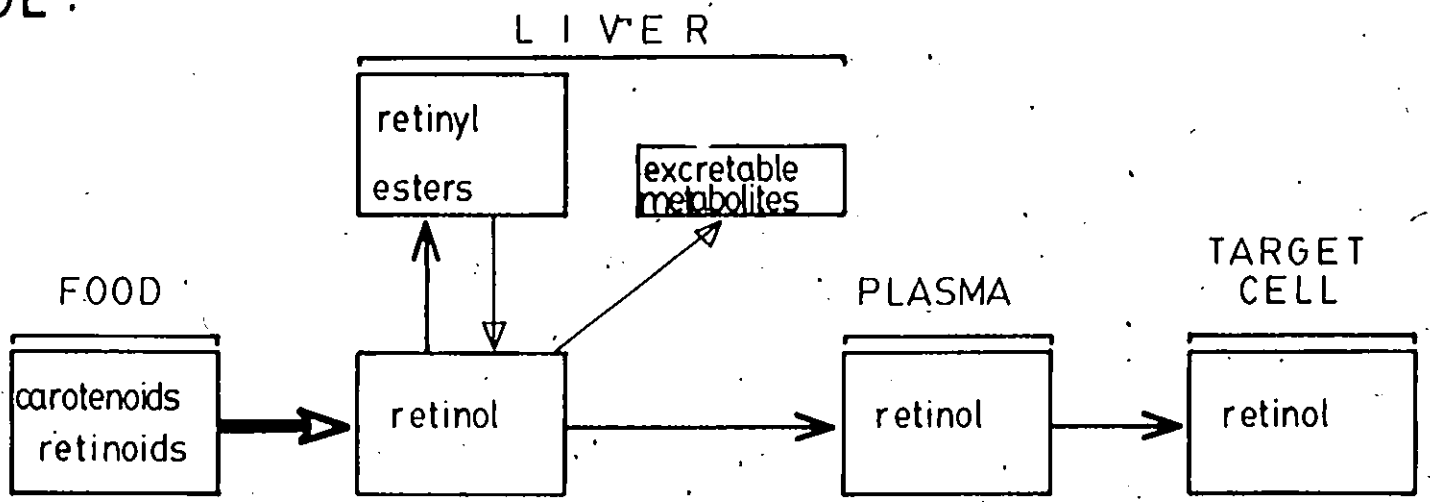

RETINYL ESTERS
RETINOL
RETINALDEHYDE
RETINOIC ACID
RETINOYL-β-GLUCURONIDE
4-HYDROXY-RETINOIC ACID
4-OXO-RETINOIC ACID

Regulatory enzymes of this pathway have yet to be identified, but the accelerated metabolism of retinoic acid may increase the flux of preceding substrates. This would explain the decrease in hepatic retinol detected in exposed ring doves, and the decreased concentrations of hepatic retinol and retinyl palmitate measured in Lake Ontario herring gulls.

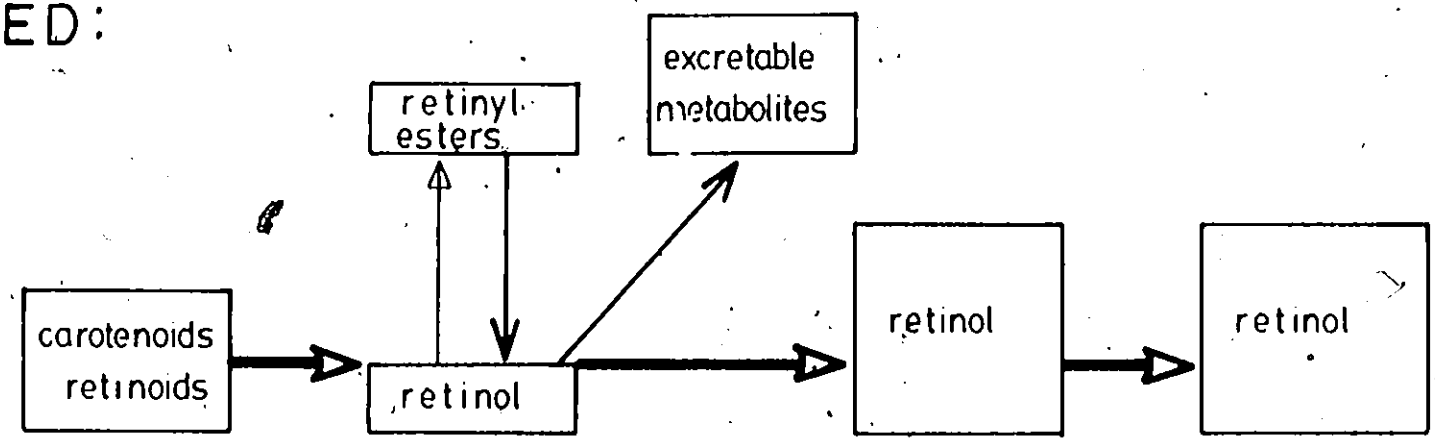
Some confusion exists in the literature with respect to the change in serum retinol following exposure to dioxin-like compounds. The present research, however, establishes that serum retinol may increase or decrease depending, in part, upon the digestive phase of the organism. Figure 26 is a model illustrating what may happen in organisms actively ingesting vitamin A compounds from a vitamin A sufficient diet. In exposed organisms, hepatic retinol and retinyl esters decrease while excretable metabolites increase according to the proposed alterations in the retinoid pathway (Fig. 25). A greater proportion of liver retinol is bound to RBP, is released into the blood, and reaches the target cells. The model explains the increased serum retinol detected in fed doves exposed to 3,4,3',4'-TCBP. The mechanism responsible for increased serum retinol may involve an estrogenic action of the toxicant resulting in increased RBP synthesis. (Dioxin compounds are capable of binding to hepatic estradiol receptors in the rat, and estradiol injections increase circulating levels of retinol (Underwood, 1984; Gail D. Bellward, University of British Columbia, pers. commun.)).

Fig. 26. Proposed model for the effects of dioxin-like compounds on retinoid homeostasis in the case of adequate dietary supply of vitamin A. Size of arrows indicates relative rates of transfer between compartments.

CONTROL:

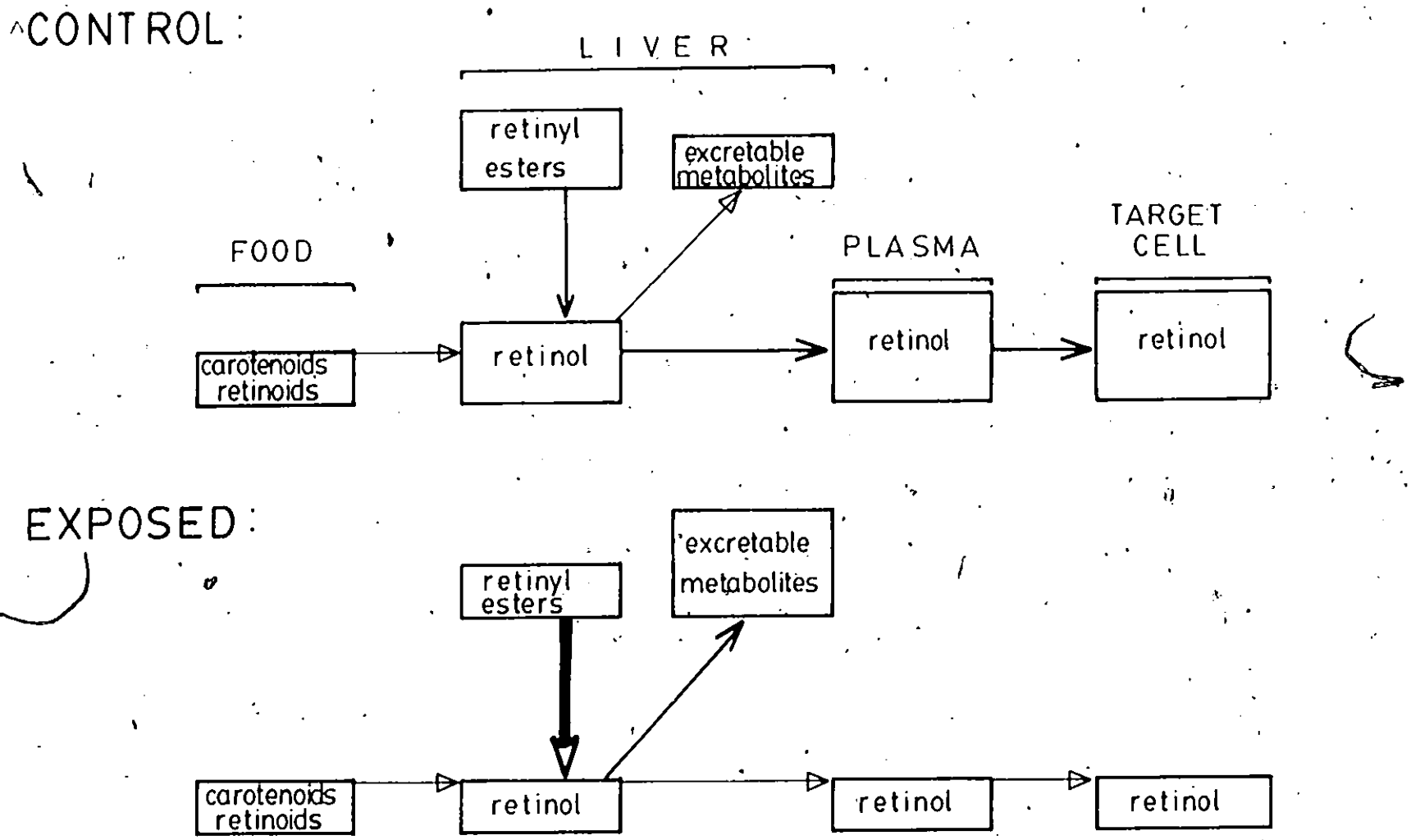


EXPOSED:



In the case of organisms which are fasting, in a post-absorptive phase of digestion, or ingesting low vitamin A diets, a different model is proposed (Fig. 27). Due to the diminished intake of retinoids, hepatic retinyl esters are mobilized to maintain normal plasma retinol concentrations. Retinyl esters are further mobilized in exposed organisms according to the effects on liver retinoid metabolism outlined in Figure 25. However, retinyl ester mobilization is presumably insufficient to maintain normal plasma retinol concentrations in the exposed organisms, and the result is a reduced vitamin supply to the target cells. This model explains the decrease in serum retinol detected in exposed poultry chicks receiving a low vitamin A-low iodine diet, and in exposed, 24 h fasted doves. The model also accounts for the quantitative relationships between enzyme induction and decreased serum retinol in 24 h fasted doves.

Fig. 27. Proposed model for the effects of dioxin-like compounds on retinoid homeostasis in the case of inadequate dietary supply of vitamin A (i.e. low vitamin A diet; fasting; post-absorptive phase of digestion). Size of arrows indicates relative rates of transfer betwen compartments.


CONTROL:
LIVER
retinyl esters
excretable metabolites
FOOD
PLASMA
TARGET CELL
carotenoids retinoids
retinol
retinol
retinol
EXPOSED:
retinyl esters
excretable metabolites
carotenoids retinoids
retinol
retinol
retinol

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