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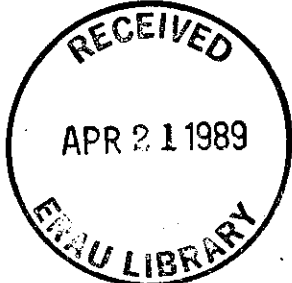
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**PROBLEMS IN
AERIAL APPLICATION:**

**I. SOME BIOCHEMICAL
EFFECTS OF LINDANE AND
DIELDRIN ON VERTEBRATES**



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**FEDERAL AVIATION AGENCY
Aviation Medical Service
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Civil Aeronautical Research Institute
Pharmacology-Biochemistry Branch
Oklahoma City, Oklahoma**

MAY, 1962

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**JACK W. DAUGHERTY, PH. D.*
DANE EUGENE LACEY, B. S.
PATRICIA KORTY, B. S.**

**Chief, Cytochemistry Section
Pharmacology-Biochemistry Branch*

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ABSTRACT

Chronic exposure to the chlorinated hydrocarbon, lindane, elicited alterations in several biochemical values of rat tissues. These included ribonucleic acid and deoxyribonucleic acid quantities, water content, and the cytochrome oxidase activity of heart sarcosomes. The chlorinated hydrocarbon, dieldrin, produced changes in the uptake of L-methionine by the particulate components of chick heart and liver cells. The specific alterations and the implications of these alterations are presented in the text.

INTRODUCTION

Despite the numerous studies that have been made on insecticide toxicology since the first use of DDT, the modes of action of even the most thoroughly studied of the newer organic agents are relatively unknown. Investigators have practically ignored this potentially fruitful area of research which has applications in industrial and aviation medicine, and animal husbandry. In addition, contributions to theoretical toxicology and biochemistry are likely. A systematic investigation of the biochemical effects of organic insecticidal agents has been initiated at the Civil Aeromedical Research Institute. It is hoped that from these studies, preventive, protective and therapeutic measures will develop which will decrease the morbidity and mortality rates in the field of aerial application.

EXPERIMENTAL

The work reported in this paper represents one part of a continuing program. The rats used in this investigation were of the Sprague-Dawley strain, as bred and supplied by Holtzman. They were obtained when 140-160 grams in weight and kept under laboratory conditions for three days before they entered an experimental regimen. The chicks were White Rocks, obtained locally when one day old, and kept under laboratory conditions until used. Commercial (Ralston) animal rations were used throughout.

The chlorinated-hydrocarbon insecticides, lindane and dieldrin, were obtained from the Nutritional Biochemicals Corporation. Adenosine-monophosphate, adenosine-diphosphate, EDTA, cytochrome-c and succinic acid were supplied by the California Corporation for Bio-

chemical Research. S-35 labeled methionine was obtained from Schwarz Bioresearch Inc.

Protein was estimated according to the method of Lowry et al (1). Nucleic acids were determined by pentose analysis; DNA by 2-desoxy-ribose (2) and RNA by ribose (3). Isolation of the nucleic acids from the tissues and the removal of lipid materials was accomplished according to the methods described by Schneider (4) and Schneider and Klug (5). Nucleic acids so measured were found to compare favorably in amount with those determined by others using different criteria. Total nitrogen was determined by standard micro-Kjeldahl techniques. The heart sarcosomes and liver mitochondria were isolated by differential centrifugation in 0.44 M sucrose. Succinoxidase and cytochrome oxidase activities were measured manometrically following the usual standardization procedures. Radio-activity measurements were made with a Sharp Low Beta counter. Chromatographic analyses were carried out on Whatman No. 4 filter paper using ascending principles with phenol and 2, 4-lutidine as the solvents. Specific procedural details are described in connection with appropriate results below.

RESULTS

The DNA Content of Rat and Chick Tissues.

In reporting results concerning the activities of enzyme systems in tissue preparations from animals differing in age, sex, nutritional state, experimentation, etc., it is of paramount importance that a proper and adequate choice of units be made for comparing and presenting the various biochemical data. Investigators use many different references for this purpose; wet weight of tissue, dry weight, total nitrogen content, protein content, etc. Each of these units may be adequate and appropriate for specific occasions, but in every case control studies are necessary as a means of demonstrating the reliability of the unit for comparison purposes. These are seldom provided. Too frequently such well recognized variables as tissue water content and the gain or loss of non-active connective tissue components are ignored.

Probably the most useful and reliable unit for the expression of biochemical activities is on the basis of the tissue DNA content. This value

allows a reasonably close correlation of activity measurements with the amount of chemically active material (cells) included in the tissue preparations. Comparisons between different tissues, tissues from different animals and tissues experimentally altered can thus be justifiably made. In addition, the increase in the significance of experimental results is worth more than the extra effort required to make the estimate of DNA. The results in Table I, showing the variability in DNA content of comparable amounts of different tissues of the rat and

TABLE I
VARIATION IN DNA WITH AGE

(Milligrams per gram wet weight)

Organ	Rat Age			Fowl Age		
	1 Wk	3 Mo	1 Yr	1 Wk	3 Mo	5 Mo
Heart	3.98	0.67	0.71	1.56	0.86	0.81
Liver	4.44	1.08	2.16	3.39	1.50	1.99
Kidney	5.84	2.02	2.28			1.79
Brain	2.34	1.32	1.22	1.81	1.40	1.28
Spleen	7.48	7.75	6.22	4.04	4.73	6.09

chick, are evidence of the advisability of making this determination. It is apparent that on the basis of DNA content, considerable differences obtain between tissues which vary as to the source and age.

Effect of Lindane Exposure on DNA and RNA. In experiments designed to study some of the biochemical effects of lindane toxicity, additional evidence was disclosed indicating the desirability of basing enzyme kinetics data on concurrently derived DNA values. Table II shows the DNA and RNA content of certain tissues following exposure of female rats to daily intraperitoneal injections of 17 mg. of lindane in paraffin oil per kg. of body weight, for a two-week period. Controls received paraffin oil only. These results are in agreement with earlier results on rats which were exposed to lindane for 31 days. In these animals there was a pronounced increase in DNA

TABLE II
EFFECT OF LINDANE ON RNA AND DNA
OF RAT TISSUES

(mg/gm dry wt)

Tissues		DNA	RNA	$\frac{\text{RNA}}{\text{DNA}}$
Heart	Control	4.24	7.97	1.88
	Test	4.52	14.20	3.14
Liver	Control	3.82	25.30	6.62
	Test	3.52	26.10	7.42
Kidney	Control	9.50	15.20	2.46
	Test	7.95	12.30	1.55
Brain	Control	4.71	8.45	1.80
	Test	4.03	8.70	2.16
Spleen	Control	40.30	20.80	0.52
	Test	58.30	40.40	0.69

of heart and spleen and a decrease in brain and kidney. Little change in DNA was observed in the liver. The spleen DNA was consistently high in quantity, and showed a remarkable increase following exposure to lindane for even relatively short periods of time. It is apparent from these data that alterations of considerable magnitude can result from the presence of lindane in the cellular environment. The extent to which these changes affect the physiology of the cell or contribute to the symptomatology of toxicity is not known.

Effect of Lindane on Heart Sarcosome Activity. From the general toxicological features of lindane poisoning on higher animals, a working theory was formulated that the physiological effects were, perhaps in some part, attributable to an effect on the active transport of important metabolites in the tissues. Since this phenomenon is energy dependent, it was reasoned that a study of the chemical reactions responsible for providing energy in a form suitable for this function was a logical starting point in an investigation of the specific biochemical effects of chlorinated hydrocarbons on higher animals.

Two often-studied oxidative enzyme systems were selected for initial study — cytochrome oxidase and succinic dehydrogenase. These are known to be linked biochemically to the synthesis of ATP in cells and tissues, and in addition, have several other relationships and characteristics which emphasize the logic of selecting them for study in the present overall research plan: Morrison and Brown (6) showed that the cytochrome oxidase activity of cockroach coxal muscle was sensitive to chlorinated hydrocarbons; both of the systems are located predominantly in the particulate fraction of the cell and in this regard are available for certain assay purposes in future studies of interface phenomena within cells; and reliable histochemical techniques are available for the study of such oxidative reactions.

Table III shows that the cytochrome oxidase activity of sarcosomes from the heart of the

TABLE III
EFFECT OF LINDANE ON SPECIFIC
ENZYME ACTIVITY OF RAT
HEART SARCOSES

(μ MO, per mg protein per hour)

Tissue*	Succinic Dehydrogenase	Cytochrome Oxidase
Control	36.4	108.0
Test	40.5	180.0

*Females exposed 2 weeks

female rat was markedly increased by lindane, given daily for 14 days, at a dosage level of 17 mg./kg. of body weight. A smaller, but significant effect was noted on succinoxidase activity. Control animals received the oil solvent only.

The Uptake of S-35 Methionine by Chick Tissues. Methionine, along with other amino acids and non-protein metabolites, is known to be selectively absorbed by the intestinal mucosa and the kidney tubules, and actively transported across tissue membranes (7). Critical experiments involving S-35 labeled methionine, were carried out to test part of the initial hypothesis concerning the mechanism of chlorinated hydrocarbon toxicity.

Twelve-week old chicks were given 42 microcuries of S-35 labeled L-methionine by the oral route. At allotted times duplicate 50 mg. samples of various tissues were extirpated, washed, and placed in stainless steel counting planchets 1 inch in diameter and 5/16 inch deep. The samples were then wet-ashed with conc. HNO₃, dried with low heat in vacuo, and their radioactivity measured. Corrections for decay and self-absorption were made.

Table IV gives the results of this study of the rate of methionine uptake by the various tissues of the chick.

TABLE IV
S³⁵-METHIONINE UPTAKE BY
CHICK TISSUES

(Counts/min/mgm dry wt)

TISSUE	1 Hr	6 Hrs	12 Hrs	18 Hrs
Mucosa	928	1010	925	1013
Blood	376	358	347	294
Liver	684	739	776	648
Lung	390	382	525	424
Brain	160	244	311	300
Spleen	820	744	1027	789
Heart	7	24	23	27
Muscle	23	93	127	302

These data show that methionine is picked up by the intestinal mucosa, blood, liver, spleen and lung at such a rapid rate that by the end of the first hour the uptake by these tissues is at a maximum or nearly so. In the case of the mucosa this is confirmation of its high metabolic activity and its known high turnover rate of metabolites.

The maximum uptake of methionine by brain was not observed until the twelfth hour. The reason for this slow rate is a subject for speculation at this time, as is the limited uptake of methionine by cardiac and skeletal muscle.

The Effect of Dieldrin on Methionine Uptake. The influence of chlorinated hydrocarbons on this biological activity was then tested. The chlorinated terpene, dieldrin, was dissolved in acetone and introduced in specified amounts

into gelatin capsules. The acetone was allowed to evaporate and the encapsulated dieldrin was fed to twelve-week old chicks in daily amounts of 20 mg./kg. for three days. At this time the animals were given, by mouth, 40 microcuries of the S-35 labeled L-methionine. At the end of six hours the hearts and livers were removed and the heart sarcosomes and liver mitochondria isolated by procedures adapted from Schneider (4) and Dounce et al (8). The six hour interval was selected as this represented the minimum time required for maximum methionine uptake by cardiac muscle. Special precautions were taken in washing the particulate fractions before they were prepared for counting the radio-activity and in all cases the final wash was, itself, counted to assure the complete removal of isotope-labeled methionine from the exterior of the particles. This final wash never materially exceeded the background count. The results are presented in Table V. The decline in the rate of methionine uptake by liver and heart tissues was found to be of significance.

TABLE V
EFFECT OF DIELDRIN (2 wks) ON S³⁵
METHIONINE TRANSPORT

Tissue		6 Hour Count
Heart*	Control	28,000
	Test	26,300
Heart Sarcosomes**	Control	880
	Test	540
Liver*	Control	760,000
	Test	640,000
Liver Mitochondria**	Control	8820
	Test	7770

*Counts/min/gm dry wt

**Counts/min/particulate fraction from 1 gm tissue

DISCUSSION

It is generally recognized that of the many consequences of chlorinated hydrocarbon intoxication in higher animals, liver involvement, as evidenced by cloudy swelling, hydropic degeneration, homogeneity of hepatic cell granules, liver enlargement, cell vacuolation, etc., is among the most consistently

observed. However, little is known of the causative factors underlying the appearance of these conditions. Available information suggests that alterations in membrane permeability and active transport mechanisms may be, at least in part, responsible for the observable histopathology. Should this suggestion find sufficient experimental support, the usefulness of these chlorinated hydrocarbons as basic research tools could surpass the practical advances that application of this information would provide in civil aviation and industrial medicine.

In connection with the studies on energy requirements for active transport, efforts so far to demonstrate a specific uncoupling effect by chlorinated hydrocarbons on phosphate esterification in the synthesis of high energy phosphate bonds (ATP) have met with technical difficulties. However, useful and interesting ancillary information has resulted.

The measurements of the nucleic acid content of tissues under normal and experimental conditions have yielded interesting findings, some new, some confirmatory, concerning the assay of biochemical functions and the activities of tissue components. One cannot fail to recognize that the actual amounts of chemically (metabolically) active material in a given tissue sample may vary markedly with the instantaneous physiological condition of the animal and of the tissue. In the present studies, unpublished findings have shown an actual alteration in the water content of certain tissues following exposure to chlorinated hydrocarbons. After 31 days on lindane, the water content of the rat heart was elevated whereas that of the liver was decreased. Annau (9) reported a rise in both DNA and RNA of liver parenchyma, but no change in the nucleic acid content of brain, following the feeding of the chlorinated hydrocarbon, aldrin, to mice for from 13 to 25 days. This is at some variance with the results of the present study, but many of the experimental conditions were different, including the test animal and the insecticide. In spite of these apparent discrepancies, it is clear that investigators in the field of toxicology, whose task includes the determination of cellular biochemical activities, cannot be too demanding in the proper choice of assay controls or in the choice of references used to express findings.

Evaluation of the significance of observed alterations in RNA and DNA content of tissues must await the solution of some of the basic problems in nucleic acid metabolism. Brown and Roll (10) point out that "... we cannot adequately describe the process by which nucleic acids are formed in the living cell", and, "many situations have been described in which the administration of a compound and the induction of a certain physiological state in an animal, is followed by alterations in the amount or in the renewal of tissue nucleic acids. ... it does not follow that they are directly implicated in nucleic acid synthesis".

The observations on the effects of lindane on oxidative enzyme activity are of interest in relation to the findings of other workers. Morrison and Brown (6) found that 10^{-4} M lindane depressed cytochrome oxidase activity of the coxal muscle of the cockroach, *Periplaneta americana*, but the exposure to 10^{-4} M lindane resulted in a transitory increase in activity. In seeming contradiction, Harvey and Brown (11) observed a marked increase in O₂ consumption of *Blattella* following the injection of toxic amounts of lindane. There is at present neither reason nor basis for comparing insecticide effects on arthropods with those on vertebrates. In fact there is to date no acceptable information indicating whether or not the various effects of most insecticides on biochemical activities are direct effects of the unmetabolized compound, direct effects of the metabolically altered compound, or indirect effects, mediated through undefined channels. Work aimed toward the solution of this problem is of foremost importance in the final description of insecticide effects.

The most impressive evidence that chlorinated hydrocarbons affect active transport mechanisms is found in the results of experiments in which L-methionine translocations were followed by the use of the radioactively-labeled compound. There can be little doubt that dieldrin, under the conditions of the tests, not only depresses methionine transport across cell membranes, but also affects its movement across the heart sarcosome interface. In the experiments reported, transport into the liver cells was reduced approximately 6%, whereas transport into the particulate fraction of the cell was reduced 39%. In examining these results

it is noteworthy that approximately 95% of the methionine (measureable) taken up by the heart sarcosomes was, at the time of assay, still in the form of the free amino acid (chromatographic analysis). Any statement in explanation of this effect on the movement of methionine must await the results of studies on the more specific biochemical effects of these insecticidal agents. Of special importance will be those studies devoted to the determination of the capacity of chlorinated hydrocarbons to uncouple oxidative phosphorylation. If this is found to be the case, the increased O₂ consumption, increased cytochrome oxidase activity, decreased rate of methionine uptake and many of the other observable effects of chlorinated-hydrocarbon intoxication would be closer to adequate explanation.

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ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. Paul W. Smith for his help in certain toxicological matters in the investigation and in the preparation of the manuscript. We also wish to thank Dr. Wallace Friedberg for the use of facilities in the Radiobiology laboratory.