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ACTION MECHANISM, METABOLISM AND MUTAGENICITY STUDIES OF

NK-592 (4-METHYL 3-N-PROPYLTHIOPHENYL 4-NITROPHENYL ETHER)

A NEW ACARICIDE
presented by

Ignatius Jusuf Hadioetomo

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ACTION MECHANISM, METABOLISM AND MUTAGENICITY STUDIES OF NK-592 (4-METHYL 3-N-PROPYLTHIOPHENYL 4-NITROPHENYL ETHER) A NEW ACARICDE

By

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ABSTRACT

ACTION MECHANISM, METABOLISM AND MUTAGENICITY STUDIES OF NK-592 (4-METHYL 3-N-PROPYLTHIOPHENYL 4-NITROPHENYL ETHER) A NEW ACARICIDE

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The acaricide NK-592 kills mites (Tetranychus urticae Koch) upon contact. In vivo toxicity studies demonstrated that NK-592 is more toxic than its oxidation product, the sulfoxide ether. However, whether NK-592 is a toxicant of the central nervous system is not clear.

The rat fed ^{14}C-NK-592 excreted most of the radioactivity in the feces. Twenty four hour post-treatment resulted in the recovery of high radioactivity from blood and excretory organs. ^{14}C-NK-592 was the major residue found in adipose tissue at 21 days post-treatment. In vivo and in vitro studies suggest that mixed-function oxidase was the major drug metabolizing system for NK-592 degradation. Several primary metabolites were identified. However, no glucuronic acid, sulfate or glutathione conjugates were found.

NK-592 is slightly mutagenic with or without metabolic activation. Its oxidation products, the sulfoxide and alcohol metabolites, were stronger mutagens without metabolic activation suggesting that they are the mutagenic principals.

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The author wishes to express his sincere appreciation to Professor Fumio Matsumura for his guidance and advice during the course of the investigations.

To my dear wife for her support, encouragement and faith - thank you.

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INTRODUCTION

Phytophagous mites are known to cause a considerable economic loss to many agricultural, forest and ornamental plants (3), and therefore it is important to study pest management strategies which include the use of acaricides to control these populations. The ever increasing incidence of resistance of spider mite to existing acaricides necessitates that new chemicals be found to replace these materials (17).

NK-592 (4-Methyl-3-n-propylthiophenyl-4-nitrophenyl ether) is a new type of specific acaricide whose chemical structure is quite different from any other chemicals being used today. March (1958) has defined specific acaricide as a compound effective against mites at dosages which are largely ineffective against insects. NK-592 is a specific acaricide since it shows no significant insecticidal properties.

Because of its unique chemical structure, it is suspected that NK-592 has a different mode of action from the acaricides currently being used. It is more toxic to eggs and nymphs of two-spotted spider mite (Tetranychus urticae Koch) than to adult mites, indicating its uniqueness.

Most organic pesticides are lipid soluble and tend to accumulate in biological systems, and some of them disrupt cellular processes. Metabolic processes that make pesticides more polar, water soluble and excretable (33), and thereby less harmful to biological systems.

The rate of disappearance of a pesticide from the whole body, therefore often can be taken as a rough estimate of the rate of metabolism (12). It is generally accepted that liver is the principal site of drug metabolizing activity in vertebrates and the enzymes concerned are located primarily in the hepatic endoplasmic reticulum (5). One of the most important metabolic reactions is the one mediated by the oxidative enzymes known as microsomal "mixed-function oxidase," as they are specifically localized in the microsomal fraction, and have a wide spectrum of substrate specificity. The main characteristics of the microsomal mixed-function oxidase are the common requirement for oxygen and NADPH (5). They are inhibited by carbon monoxide (9), pyrethrin synergists such as sesamex, piperonyl butoxide, sulfoxide and propylisome (32), and also SKF-525A(4). Microsomal mixed-function oxidase system has been chosen as the study object for the degradation of NK-592.

Chemicals that exhibit mutagenic activity pose a potential hazard to the users, and there is an increasing acceptance of mutagenicity testing as an integral part of a toxicological evaluation of various chemicals (28). In 1975 Dr. Bruce N. Ames of University of California-Berkeley, developed a quick method for screening the mutagenic activity using specially developed strains of Salmonella typhimurium. By using this technique he has shown at 85% of the chemical carcinogens are mutagens. Despite some criticisms on its accuracy in forecasting carcinogenicity of many chemicals, it is certain that the test reveals mutagenic activity which by itself should signal potential danger of the chemical.

The purpose of using the Ames test in this work was to examine the future potential danger of NK-592, a newly developed compound. It must be clearly understood that a positive result in Ames test does not signal an immediate danger. It merely points out the necessity of further examination for carcinogenicity test such as *in vivo* chronic test.

MATERIALS AND METHODS

Materials

Animals

The rats used throughout the study were young adult male Sprague-Dawley rats weighing 150-200 g. They were obtained from Spartan Research Animals, Inc., Haslett, Michigan.

Insects and Plant Material

Two strains of the two-spotted spider mites (*Tetranychus urticae* Koch) were used.

1. The "Greenhouse Resistant Strain" that are resistant to azinphos methyl. Unless otherwise stated mites of this strain were used throughout the study.
2. The susceptible strain was the New Zealand strain derived from the susceptible LN4 strain, Lincoln College, Canterbury, New Zealand.

Both strains were kindly provided by Dr. Brian A. Croft, Pesticide Research Center, Michigan State University, East Lansing, Michigan. They were reared on string bean (*Phaseolus vulgaris*) var. harvester.

Bacterial Strain

Salmonella typhimurium strain TA 100 was kindly provided by Dr. Bruce N. Ames, Biochemistry Department, University of California-Berkeley.

Chemicals, Biochemicals and Others

^{14}C-NK-592 (specific activity 4.61 mCi/mmole), unlabeled NK-592, and 16 candidate metabolites were provided by Nippon Kayaku Co., Ltd., Tokyo, Japan. Dicofol technical grade was obtained from Rohm and Haas Company, Philadelphia, Pennsylvania. Guthion® 50 W.P. (azinphos methyl) was obtained from Chemagro Agricultural Division, Kansas City, Missouri. Biochemicals such as nicotinamide adenine dinucleotide phosphate (NADP), reduced nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-phosphate (G-6-P), reduced glutathione (GSH), uridine-5'-diphosphoglucuronic acid (UDPGA), adenosine-5'-triphosphate (ATP), adenosine-5'-phosphosulfate (APS), bovine albumin, β-glucuronidase type B-3, sulfatase type VIII, ampicilin, benzo [a] pyrene, histidine, d-biotin, dithiothreitol (DTT), N-ethylmaleimid(NEM), p-chloro mercuribenzoate (PCMB) and Folin & Ciocalteu's phenol reagent were purchased from Sigma Chemical Company, St. Louis, Missouri. 2-Amino anthracene was purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. 4-Nitroquinoline-N-oxide was purchased from ICN Pharmaceuticals, Inc. Plainview, New York. Sesamex was obtained from Shulton, Inc., New York. SKF-525A was obtained from Smith Kline & French Laboratories, Philadelphia, Pennsylvania. Liquid scintillation phosphors, PPO and dimethyl POPOP, and Triton X-100 were purchased from Research Products International Corporation, Elk Grove Village, Illinois. Silica gel came from E. Merck, Germany. Analytical TLC precoated plates LK5F from Whatman Inc., Clifton, New Jersey and no screen X-ray films from Eastman Kodak Co., Rochester, New York. Bacto agar and nutrient broth were purchased from Difco Laboratories, Detroit, Michigan. All

solvents were glass distilled grade and purchased from Burdick and Jackson Laboratories, Muskegon, Michigan. Other chemicals were of reagent grade that were available locally.

Methods

Studies on the Action Mechanism of NK-592 on Mites

Preparation of acaricide for biological essay

Pure NK-592, sulfoxide ether and dicofol do not dissolve in water. For biological tests a 20% emulsifiable concentrate was prepared by mixing 200 mg crystals with 750 µl toluene and 100 µl Triton X-100 to make the total volume of 1.0 ml. Azinphos methyl used was the 50% wettable powder formulation. A mixture of 900 µl toluene and 100 µl Triton X-100 was similarly prepared as a control. Upon mixing with water an emulsion was produced, and this emulsion was used for spray operations or slide dip experiments.

Determination of lethal concentration (LC) against mites by various methods of application

In this study adult female mites were used, because of the convenience in handling. They are larger than the males, more abundant and more tolerant of rough handling.

In most cases the test was conducted on a 2 x 2 sq cm bean leaf and using 25 healthy mites with 5 replicates. Each leaf square was sprayed at a 25 cm distance from the spray nozzle of a Thomas-John spray bottle. They were placed in petri dishes whose bottoms were lined with moistened filter paper. The excess water on the

leaf surface was allowed to dry before the petri dishes were covered. The mortality of the mites was observed 24 hour post-treatment. Mites were considered dead when no leg or body movements were observed upon prodding with a camel hair brush.

To determine the LC upon direct spraying, mites were transferred to the leaf squares before the spray operation, while in determining the LC of its residue, mites were transferred after the spray operation.

The slide dip method (34) was adopted in determining the LC of those acaricides with slight modifications as follows: dipping time was 30 seconds and mortality was observed 24 hour post-treatment.

Determination of lethal time (LT) by slide dip method

The emulsion used for dipping contained acaricide at the LC_{90} dose as determined by the previous experiment. Other treatments and handling were the same as those described above.

Determination of LC against mites at different stages of growth and sex by direct spray application

In this experiment, susceptibility of females, males, nymphs as well as eggs were examined. Eggs were obtained by transferring 5 adult females to each leaf square and allowing them to lay eggs. After 2 days the number of eggs deposited were sufficient and the adults were removed before spraying. Mortality was observed as described previously, except for ovicidal activity which was carried out 1 week post-treatment by observing the number of unhatched eggs.

Processing of LC
and LT data

Correction for natural mortality were made using Abbott's formula (1). Logarithm-probit method was adopted to estimate the LC_{50} and LT_{50} (11).

Study on the systemic
property of NK-592

Three healthy growing young plants with 2 leaves each were transferred to 3 containers filled with 50 ml each of distilled water containing NK-592 at a concentration of 1.0 ml/l water. Twenty five healthy mites were transferred to 5 of the 6 leaves. A control was prepared in the same manner except that the water did not contain NK-592. The number of dead mites were recorded every other day for 10 days. To avoid possible photodecomposition of NK-592, the containers were wrapped with aluminum foil. Petroleum jelly was smeared on each petiole to prevent the mites from escaping.

Studies on the effect of NK-592
on mites behavior

Effect on mites migration. The undersurface of 5 out of 6 leaves on 3 healthy growing young plants were sprayed with emulsion containing NK-592 at a concentration that gave approximately 30% mortality (5.5 ppm). Excess water was allowed to dry, and 25 healthy mites were then transferred to the treated undersurface. Petroleum jelly was applied as described above. A control was prepared in the same manner without NK-592. The number of mites that moved to the upper surface of those leaves were recorded every day for 5 days.

Effect on mites dispersion. One out of 2 leaves of 5 healthy

growing young plants were sprayed with NK-592 as described above. Twenty five healthy mites were transferred to the treated leaves. Petroleum jelly was smeared on the stem. The number of mites that moved away from the treated leaves to the healthy one were recorded as described above.

Pharmacokinetics of ^{14}C-NK-592 in Rats

Treatment of rats with ^{14}C-NK-592

Five rats were conditioned individually for 24 hours in metabolism cages. They were given Purina Lab Chow and tap water ad libitum. On the next day they were given a single oral dose of ^{14}C-NK-592 (0.461 mCi/mmole) at 400 mg/kg body weight, dissolved in 0.75 ml corn oil via stomach tube and followed by a rinse with 0.25 ml corn oil. They were then kept in individual cages as described earlier.

Radioactivity counting

Radioactive metabolites were partitioned in several fractions and therefore needed different methods to determine their activity with high accuracy. Radioactivity found in organic solvents were determined by mixing the sample with 10 ml non-aqueous scintillation cocktail consisting of 5.0 g PPO, 0.35 g dimethyl POPOP and 1.0 l toluene. Radioactivity in aqueous phase was determined using aqueous scintillation cocktail consisting of 5.5 g PPO, 0.3 g dimethyl POPOP, 0.5 l toluene and 0.5 l methyl cellusolve. Radioactivity bound to silica gel was determined using a different cocktail that consists of

5.0 g PPO, 0.50 g dimethyl POPOP, 40 g Cab-O-Sil (Packard Instrument Company) and 1.0 l toluene. Radioactivity found in fecal material, body organs and tissues was determined by using Harvey Instruments OX-200 biological oxidizer whereby the ^{14}C was determined as $^{14}CO_2$ that was trapped in a cocktail consisting of 1 part Carbosorb II and 2 parts Permafluor V (Packard Instrument Company). Those vials were then counted for their radioactivity in a Searle Isocap 300 liquid scintillation spectrometer. Corrections for radioactive quenching were made by the external standard method.

Excretion study of radioactive chemicals

For the 20 days excretion study, urine and feces samples were collected separately from 3 individual rats at regular intervals.

Each time a sample of urine was measured and adjusted to certain volume for accuracy. Aliquots were taken for radioactive counting. Each sample of feces collected was air dried, ground and weighed. A known amount of those samples was used for radioactive determination as described above.

Distribution and storage of radioactive chemicals in various body organs and tissues

Two treated rats were sacrificed 24 hour post-treatment by cerebral concussion. Various organs and tissues were dissected out immediately and known amounts of them were sampled for radioassay.

After 20 days the remaining rats were sacrificed and the radioactivities in various organs and tissues were examined as described above.

Analysis of body fat

A small amount of body fat was blended with 25 ml acetonitrile for about 1 minute on a Sorvall omni mixer, and poured into a 500 ml separatory funnel. This procedure was repeated once more to make sure that all fat tissue were dissolved and left only the connective tissues then the 2 acetonitrile extracts were combined. One hundred ml of 10% sodium chloride solution and 100 ml of methylene chloride were added and the solution shaken vigorously for about 1 minute. Two layers were formed, the solvent was the bottom layer. The water layer was discarded and another 100 ml 10% sodium chloride solution was added to wash the acetonitrile. The layers were again separated and the remaining solvent evaporated to a small volume using a rotary evaporator and then analyzed by TLC.

Metabolism studies

In vivo studies

Extraction and analysis of feces. The general scheme of extraction and analysis is summarized in Fig. 2 (p. 32). The combined air dried feces was transferred to an Erlenmeyer flask and acetone (20 ml/g) was added. The flask was shaken on a Burrel wrist action shaker for 30 minutes. The extraction was repeated twice, and all 3 extracts were combined and evaporated on a rotary evaporator to a small volume for radioassay. This extract was evaporated to dryness leaving an oily residue. Hexane was added to dissolve the hexane-soluble metabolites. The hexane-insoluble fraction was subjected to partitioning 3 times with acetonitrile (1:1 v/v). The acetone fraction was again evaporated to dryness

and the dry residue was subjected to partitioning between water and ethyl acetate. The remaining dry feces after acetone extraction were extracted again 3 times, each time with 20 ml/g of methanol. All 3 methanol extracts were combined and evaporated to a small volume for radioassay. This fraction was evaporated to dryness and the residue was partitioned between distilled water and ethyl acetate. A radioassay was performed for each fraction in every extraction step. The remaining dry feces after methanol extraction were weighed and small samples were taken for radioassay by combustion. The same procedure was applied to the air dried ground feces before extraction to determine the initial radioactivity.

The acetonitrile fraction, ethyl acetate fraction from the acetone extract and ethyl acetate fraction from the methanol extract were further analyzed by thin-layer chromatography (TLC) and autoradiography. For quantitation of the radioactive metabolites, the silica gel portions corresponding to the dark spots on the autoradiogram were scraped and counted. Further identification of the metabolites were carried out by both TLC and gas-liquid chromatography (GLC).

To study the nature of the water soluble metabolites, the aqueous fraction of the methanol extract was analyzed as follows: 0.5 ml extract was incubated either with 7,000 units β-glucuronidase or 150 units sulfatase in 3.5 ml 0.2 M acetate buffer at pH 5.0 or with 3.5 ml dilute HCl or 3.5 ml buffer only to serve as control. The reaction mixtures were incubated at 37^{o}C for 24 hours. The reaction was terminated by adding 4.0 ml ether and extracted directly. Extraction was repeated twice and all 3 extracts were combined for analysis of radioactivity.

Extraction and analysis of urine. The general scheme of extraction and analysis is summarized in Fig. 3 (p. 33). The combined urine samples were saturated with NaCl and the pH was adjusted to 3.0 with HCl. The solution was extracted 3 times with ethyl acetate (3.0 ml/ml) in a separatory funnel. All 3 extracts were combined and evaporated to small volume. This extract was evaporated to dryness leaving an oily residue. Hexane was added first to dissolve the soluble metabolites, followed by ethyl-acetate to dissolve the remaining hexane-insoluble ones. The hexane-soluble fraction was subjected to partitioning with acetonitrile for 3 times. The ethyl acetate fraction was evaporated to dryness and the dry residue was subjected to partitioning between distilled water and ethyl acetate. The amount of radioactivity was determined for each fraction obtained in every extraction step.

The last ethyl acetate fraction was analyzed by TLC and GLC. The nature of metabolites in the urine after ethyl acetate extraction and aqueous phase of the hexane-insoluble fraction was studied by treating them with deconjugation enzymes as described in the previous section for fecal metabolism.

Thin-layer chromatography (TLC). For quantitative study, Whatman LK5F precoated plates with 250 μ silica gel layer were used. Feces and urine extracts were spotted side by side with the candidate metabolites and developed in a mobile phase which consists of hexane-chloroform-acetonitrile (12:8:11). The radioactive spots were detected by autoradiography. Collection and further identification were carried out on laboratory prepared TLC plates developed in different mobile phases (Appendix II). Here radioactive spots were

located by Berthold TLC Scanner model LB 2760. Their R_f values were compared to the R_f values of the candidate metabolites.

Gas-liquid chromatography (GLC). For further confirmation of the nature of metabolites, a Varian Aerograph series 2400 gas chromatograph equipped with alkali flame ionization detector was used. The columns were 3% SE-30, 3% OV-101 and 3% QF-1, all on Gas Chrom Q 80-100 mesh. The SE-30 was packed in a glass column 6 ft long and with a 2 mm I.D., whereas the other 2 were packed in nickel columns of 6 ft long and with a 1/8" O.D. Operating conditions were: injector temperature 270°C, detector temperature 270°C, nitrogen carrier gas flow 30 ml/min, air flow 235 ml/min and hydrogen gas flow 48 ml/min. Identification was carried out by comparing the retention time of the metabolites to those of the candidate metabolites.

In vitro studies

Preparation of liver homogenate. In this study 3 types of liver homogenate preparations were used: 20,000 x g supernatant (i.e. crude supernatant), 100,000 x g microsomal fraction and 100,000 x g supernatant. The method for preparation of these subcellular fractions has been described by Conaway et al. (8).

Incubation of ^{14}C-NK-592 with liver homogenate. In a typical experiment 0.05 μmole (0.2305 μCi) of ^{14}C-NK-592 in 10 μl methyl cellusolve was incubated with 0.5 ml of either crude supernatant, 100,000 x g supernatant or 100,000 x g microsomal fraction plus various combinations of cofactors in a 25 ml Erlenmeyer flask. The required cofactors such as GSH, NADPH, UDPGA, ATP, APS and $MgCl_2$ were dissolved in the standard buffer (0.15 M sodium phosphate pH 7.4 and 0.05 M

sucrose) at desired concentrations. By adding standard buffer, the incubation mixture was finally made up to 2.0 ml. In some cases inhibitors dissolved in standard buffer were added to the system at the same time as the addition of the substrate. The reaction mixtures were incubated aerobically at 37°C by using a Lab-line shaking water bath for 2 hours. At the end of the incubation period 3.0 ml ether was added to stop the reaction, and the system was immediately shaken and transfered to a screw cap culture tube and extracted. Extraction was repeated 2 more times and all 3 ether extracts were combined for analysis. The control was prepared by incubating ^{14}C-NK-592 with 0.5 ml liver homogenate and 1.5 ml standard buffer without any cofactor or inhibitor. The radioactivities in both the ether and aqueous phase were determined.

Analysis of ether extractable metabolites. The combined ether phase was evaporated to a small volume (approximately 0.2 ml) under a stream of nitrogen gas and analyzed by TLC in a mobile phase which consists of hexane-chloroform-acetonitrile (3:2:2). Autoradiography was prepared and those spots having radioactivity were quantitated. Further identification was carried out by TLC and GLC as described before.

Analysis of water soluble metabolites. The aqueous phases accumulated from several control and NADPH fortified incubation mixtures were concentrated to a small volume approximately 2.0 ml using a Virtis freeze dryer. It was divided into 4 parts and further studied by using deconjugation enzymes as described in the in vivo section.

Mutagenicity Test

Bacterial tester strain

There are a number of *Salmonella typhimurium* tester strains containing different types of histidine mutations. In the experiments conducted here the strain TA 100 was used, it can detect mutagens that causes base-pair substitutions similar to strain TA 1535. In addition, this strain has a resistance transfer factor (R factor) that makes it more sensitive to a number of mutagens that could not be detected by strain TA 1535.

Preparation of mutagens

NK-592 acaricide and its candidate metabolites are not soluble in water, therefore they were dissolved in dimethyl sulfoxide (DMSO). This small amount of DMSO (less than 0.5 ml) per plate did not interfere with mutagenesis or microsomal activities. These solutions were kept sterile at all times.

Mutagenesis assay on plates

The method used to carry out this test was identical to the one described by Ames *et al.* (2).

RESULTS

Studies on Action Mechanisms of NK-592 on Mites

Lethal Concentration of NK-592 as Determined by Various Methods

Throughout the experiment the volume of spray was set at 0.2 ml of the emulsion per leaf square. The results of 3 mortality tests were summarized and are shown in Table 1. In the first 2 methods, 2 different actions can lead to the death of these mites: first the direct contact of mites with acaricide through the mist and the residues on leaf surface, and second the possible ingestion of the acaricide residue on leaf surface or dissolved acaricide in phloem or any other parts ingested by mites. In that case it acts as an oral poison. From the data it appears that the contact action is more important in the case of NK-592, because, in direct spray, the amount of acaricide that came in contact with the body is expected to be high as compared to the residue treatment. Thus the former spray method should be more efficient (i.e. lower LC_{50} figure) in producing mortality. This conclusion was further supported by the result of slide dip experiment, since in this method the mites do not have opportunities to ingest the acaricide. The fact that the LC_{50} figure was lowest in the slide dip method (i.e. most efficient mode of killing) clearly indicates the importance of direct contact and thereby establishes that penetration of this acaricide through the cuticle as the major route of entry to the mite body.

Table 1. Toxicity[a] of NK-592 Applied by Three Different Methods Against Adult T. urticae

Method of application	LC_{50}[b] (ppm)	LC_{90}[b] (ppm)
Direct spray	7.73 ± 0.68 (5)	14.66 ± 0.69 (5)
Residual action	14.08 ± 2.91 (5)	22.05 ± 2.41 (5)
Slide dip	3.10 ± 0.19 (5)	6.20 ± 0.29 (5)

[a]Toxicity data 24 hour post-treatment are expressed in ppm of active ingredient.

[b]Each figure represents the average ± SE.

Variation of Toxicity of NK-592 to Mites According to Differences in Sex and Life Stage

NK-592 was found to be more toxic to male mites than female mites (Table 2). Nymphs and eggs were found to be highly susceptible to this acaricide. However, it must be mentioned here that these doses are not expressed in the actual amount of acaricide given per unit weight of these mites and eggs. In other words, since the females have the largest body weight in the two-spotted spider mite colony, it is possible that they are capable of tolerating a larger amount of acaricides.

Lethal Time of NK-592 as Determined by the Slide Dip Method

The acaricide did not kill the mites immediately; it required some time to manifest its toxicity probably due to the time needed to penetrate into the cuticle and reach the target tissue. Table 3 shows that NK-592 needed about 10 hours to kill 50% of the population, while dicofol needs only about 1.4 hours.

The Toxicity of Various Acaricides Against T. Urticae

In this experiment, both the susceptible and resistant strains were used. To examine both strain characteristics, the mites were subjected to the slide dip method by using 3 different acaricides. The results are shown in Table 4. They clearly indicate that the resistant strain is very resistant to azinphos methyl. Both strains are more susceptible to NK-592 compared to dicofol; this shows the uniqueness of NK-592.

Table 2. Toxicity[a] of Emulsion Containing NK-592 at its LC_{50}[b] Applied as Direct Spray to Eggs, Nymphs, Adult Females and Adult Males of *T. urticae*

Sex and life stage	Percent mortality
Adult males	68.0 ± 10.3 (5)
Adult females	33.6 ± 9.7 (5)
Nymphs	100.0
Eggs	100.0

[a]Figures for both adult males and females represent the average ± SE; assessments were carried out 24 hour post-treatment.

[b]LC_{50} for direct spray is 7.73 ppm.

Table 3. Effects of Various Length of Post-Treatment Exposure Time on the Mortality of T. urticae as Determined by Slide Dip Method

Acaricide	LT_{50}(hr.)[a]	LT_{90}(hr.)[a]
NK-592	10.06 ± 0.57 (5)	24.12 ± 2.60 (5)
Dicofol	1.40 ± 0.21 (4)	14.36 ± 4.80 (4)

[a]Each figure represents the average ± SE.

Table 4. Toxicity[a] of Various Acaricides Against T. urticae as Determined by Slide Dip Method

	Acaricide		
	NK-592	Dicofol	Azinphos methyl
Greenhouse resistant strain			
LC_{50}	3.10 ± 0.19 (5)	14.03 ± 0.43 (5)	> 8 x 10^3
LC_{90}	6.20 ± 0.29 (5)	22.79 ± 0.45 (5)	-
Susceptible strain			
LC_{50}	6.61 ± 0.45 (4)	17.63 ± 0.42 (4)	389.61 ± 97.71 (5)
LC_{90}	9.90 ± 0.81 (4)	41.66 ± 7.50 (4)	988.30 ± 646.25 (5)

[a]Toxicity data are expressed in ppm of active ingredient, assessment were carried out 24 hour post-treatment.

Each figure represents the average ± SE, except the LC_{50} of azinphos methyl against the greenhouse resistant strain.

The Toxicity of the Sulfoxide Metabolite and the Effect of Sesamex Addition to NK-592

Some chemicals can by themselves be toxic but others need metabolic activation, usually oxidation. A good example is systox. In the case of NK-592, it seems that the parent compound itself is more toxic than the sulfoxide, the major oxidative metabolite (Table 5). The addition of sesamex, known to inhibit the mixed-function oxidase (32) and thereby expected to reduce the formation of sulfoxide, did increase the toxicity of NK-592 significantly. This finding supports the above theory that it is NK-592 itself that is toxic to mites.

Observation of Toxic Symptoms of NK-592 on Mites

Different group of acaricide will produce different toxic symptoms, depending upon its action mechanism. It is known that most insecticides and acaricides act upon the nervous system of the insect. Upon observation of the NK-592 produced symptoms of mites it appeared that they become less mobile and showed signs of ataxia accompanied by tremors. It could be possible that the decrease in mobility was caused by ataxia. No signs of convulsions, that are usually produced by organophosphorous compounds, were observed. The symptoms were, however, similar to that produced by the acaricide dicofol, which has a similar chemical structure to the insecticide DDT. Most of the dead mites have their legs stretched out rigidly in front of their body resulting in high carriage. There were no signs of dessication of the dead mites even after a few days.

Table 5. Toxicity[a] of the sulfoxide metabolite and the effect of sesamex addition to NK-592 emulsion

Treatment	LC_{50} (ppm)[b]		% mortality[c]
	A	B	
Slide dip method			
NK-592	3.10 ± 0.19 (5)	6.61 ± 0.45 (4)	
Sulfoxide metabolite	>20 (5)	>40 (5)	
Spray method			
Sesamex			0
NK-592[d]			65.0 ± 9.2 (5)
NK-592 + Sesamex[e]			87.0 ± 2.5 (5)*

[a]Toxicity data 24 hour post-treatment, expressed in ppm of active ingredient and each figure represents the average ± SE.

[b]The test was conducted using both the resistant and susceptible strain. A - Greenhouse resistant strain. B - New Zealand strain.

[c]The test was conducted using the Greenhouse resistant strain only.

[d]The concentration of NK-592 used was 10 ppm.

[e]The amount of sesamex added was 10 times the amount of NK-592.

*Significantly higher than NK-592 only at $P \leq 0.05$

Studies on the Systemic Action of NK-592

NK-592 was found to have no systemic action as judged by the lack of the mites mortality when the acaricide was mixed with the water at the base of the plant.

Effect of NK-592 on Migration and Dispersion Behaviour of Mites

In this series of experiments it was established that NK-592 did not cause the mites to move away from the treated area. Nor was there a tendency for the mites to migrate to the upper surface of the leaves that were not treated. At the end of the experiment most of the mites were still on the treated area (Table 6). In migration tests, the percentage of mites that moved away from the treated area were significantly lower than the control; this might be because of certain degree of toxicity that makes them less mobile. In general NK-592 did not increase or decrease locomotive activities at the doses utilized.

Pharmacokinetics of ^{14}C-NK-592 in Rats

Excretion of ^{14}C-NK-592 and Metabolites

For the first 12 hour post-treatment, more radioactivity was excreted in the urine than feces. However, most of the radioactivity was excreted in the feces thereafter. The excretion of radioactivity in the feces and urine was approximately 56% and 27% respectively. From the data shown in Fig. 1, only 83% of the total administered radioactivity was excreted.

Table 6. The Percentage of Mites that Moved Away from the Treated to the Untreated Area

	Control[b]	NK-592[b]
Migration test[a]		
1st day	42.4 ± 7.3 (5)	25.8 ± 4.3 (5)
2nd day	65.6 ± 7.6 (5)	37.8 ± 4.0 (5)*
3rd day	63.4 ± 3.1 (5)	46.2 ± 4.4 (5)*
Dispersion test[a]		
1st day	9.0 ± 3.0 (5)	3.4 ± 1.7 (5)
2nd day	9.0 ± 3.0 (5)	9.5 ± 2.6 (5)
3rd day	10.7 ± 2.3 (5)	9.5 ± 2.6 (5)

[a]See the method section for the details of the experimental design.

[b]Each figure represents the average ± SE.

*NK-592 treatment resulted in a significantly ($P < 0.05$) lower percentage of mites that moved away from the treated area.

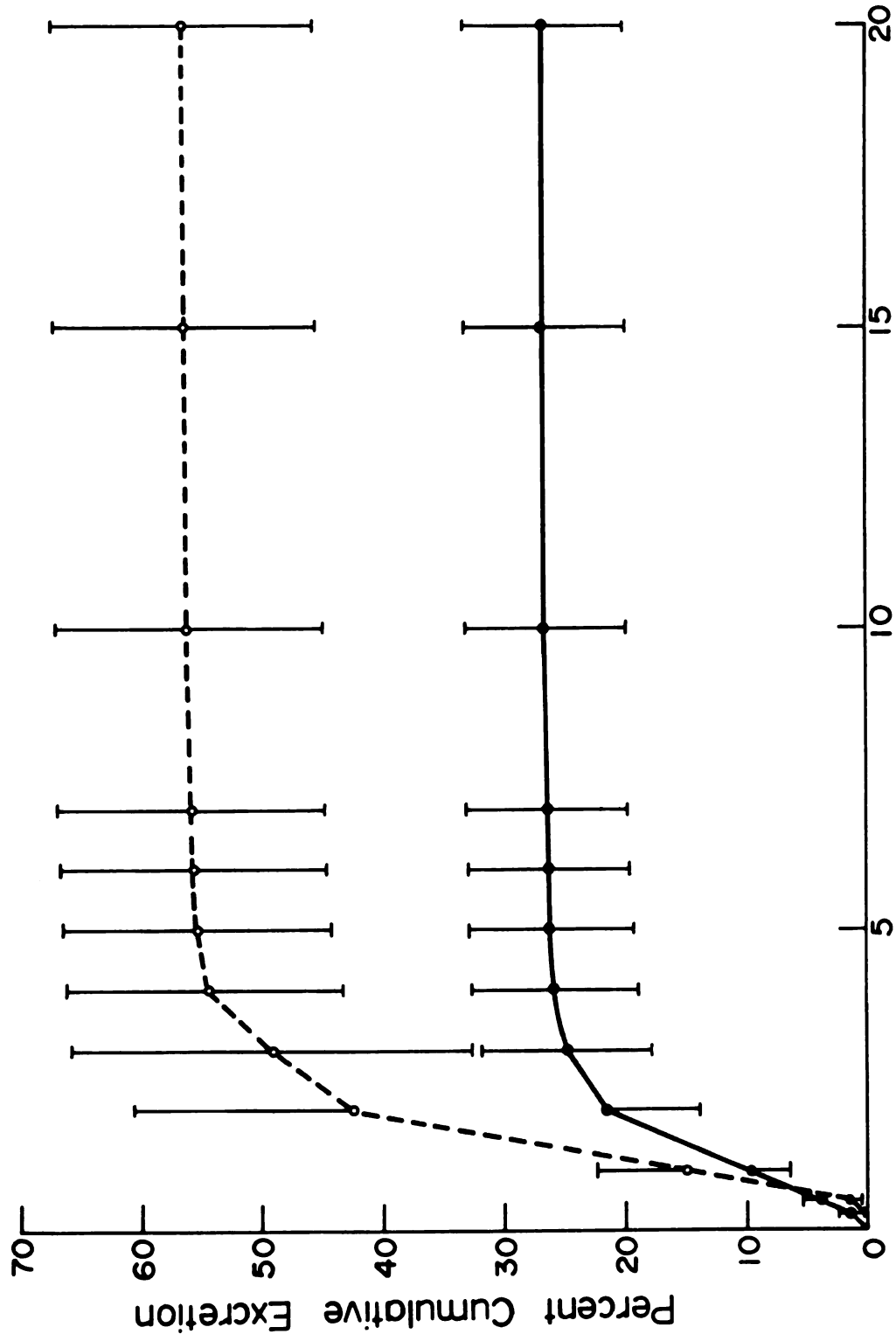


Fig. 1. Cumulative excretion of radioactivity in feces (o--o) and urine (o——o) of rats (average of three rats).

Distribution of Radioactivity in Different Organs and Tissues

The bulk of the ingested radioactivity was excreted in both the feces and urine, and a small percentage was retained by the body in various organs and tissues. After ingestion of the acaricide, NK-592 must go directly into the gastrointestinal tract and be absorbed by the blood, then carried to the liver and subsequently circulated throughout the body. Therefore, it is not surprising that relatively high levels of radio activities were retained in those organs. From the results shown in Table 7 it is apparent that the level of radioactivity at 24 hour post-treatment was highest in the blood, the excretory organs, and storage tissue such as adipose tissue.

Storage of Radioactive Chemicals in Various Body Organs and Tissues

Most pesticides, especially the apolar compounds such as chlorinated hydrocarbons, tend to accumulate in tissues that have relatively high content of lipids such as adipose tissue. It is therefore not surprising that the amount of radioactive chemical was high in the adipose tissue 21 days post-treatment as can be seen in Table 8. It is noteworthy that the level of radioactivity was also high in the spleen, kidney and liver.

Residue of Chemical in Body Fat

From the TLC analysis using mixtures of hexane-chloroform methanol (4:5:1) and hexane-ether-acetone (2:1:1) it was shown that the radioactive chemical residue bound to the fat tissue was NK-592 itself. This was not surprising because it was the least polar

Table 7. Distribution[a] of radioactive chemicals in different body organs and tissues 24 hr. post-treatment.

Type of organ or tissue	Concentration of radioactive chemicals (ppm)	Percentage radioactive chemicals from amount administered
Brain	0.4069 (0.3513 - 0.4624)	8.0×10^{-7} (7.0 - 9.0×10^{-7})
Lung	2.5511 (2.4412 - 2.6610)	51.5×10^{-7} (49.0 - 54.0×10^{-7})
Heart	1.1541 (0.9268 - 1.3813)	16.5×10^{-7} (13.0 - 20.0×10^{-7})
Liver	6.1790 (5.0158 - 7.3421)	516.5×10^{-7} (419.0 - 614.0×10^{-7})
Kidney	5.2367 (4.4296 - 6.0437)	142.0×10^{-7} (120.0 - 164.0×10^{-7})
Spleen	0.6208 (0.5394 - 0.7021)	4.0×10^{-7} (3.0 - 5.0×10^{-7})
G.I. Tract	6.1516 (4.7459 - 7.5552)	386.5×10^{-7} (298.0 - 475.0×10^{-7})
Adipose tissue	5.8497 (3.0938 - 8.6055)	$1,341.5 \times 10^{-7}$ (711.0 - $1,972.0 \times 10^{-7}$)
Muscle	0.5963 (0.5485 - 0.6440)	629.5×10^{-7} (578.0 - 681.0×10^{-7})
Blood	7.7010	$1,290.0 \times 10^{-7}$

[a]Each figure are mean of two rats, except for the blood sample.

Table 8. Amount of Radioactivity[a] in Different Body Organs and Tissues 21 Days Post-Treatment

Type of organ or tissue	Concentration of radioactive chemicals (ppm)
Brain	0.0189 (0.0083 - 0.0294)
Liver	0.0572 (0.0420 - 0.0724)
Kidney	0.0579 (0.0306 - 0.0852)
Spleen	0.1077 (0.0360 - 0.1794)
G.I. Tract	0.0132 (0.0039 - 0.0224)
Adipose Tissue	0.0832 (0.0817 - 0.0847)
Muscle	0.0133 (0.0104 - 0.0161)

[a]Each figure are mean of two rats.

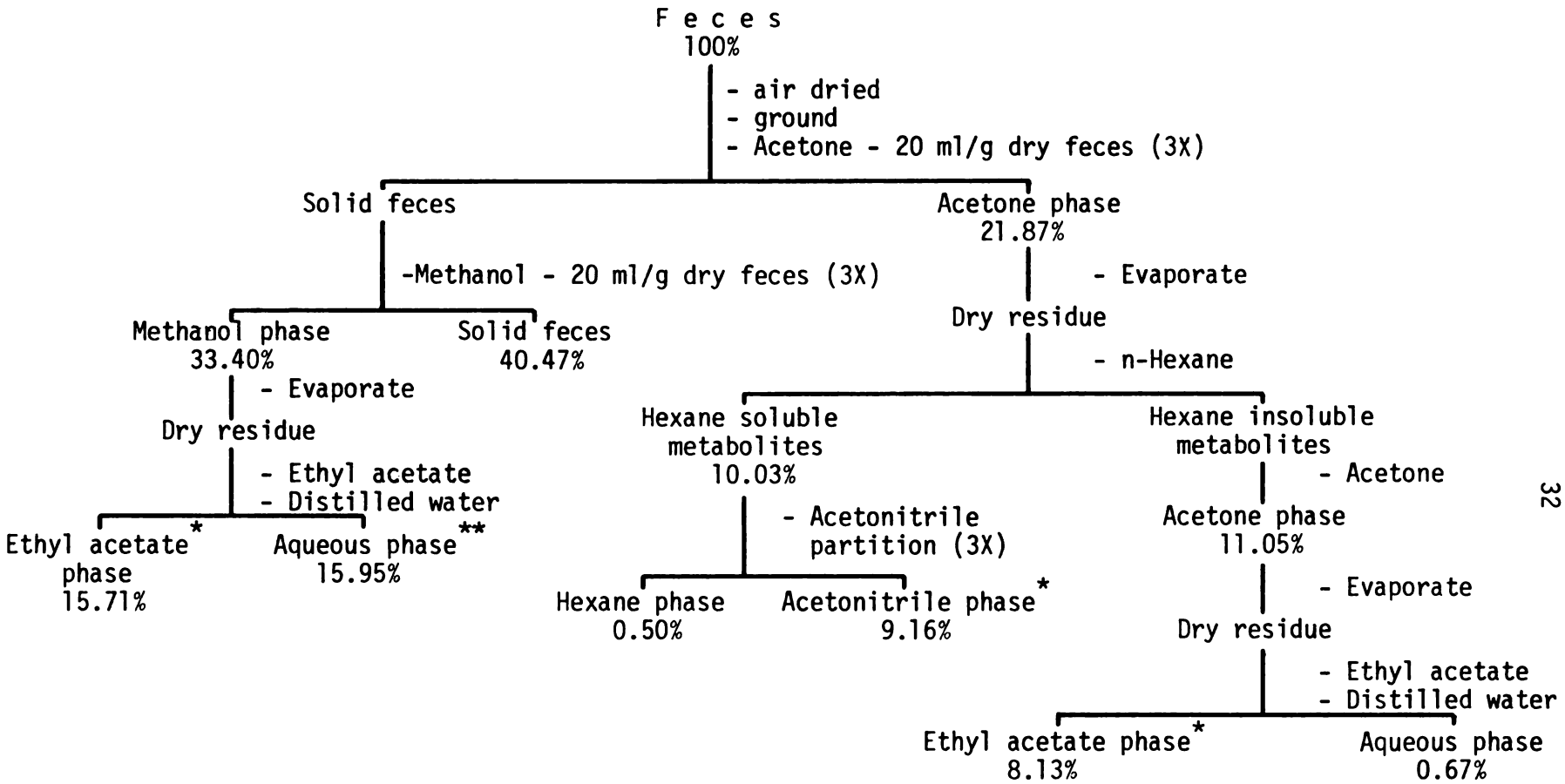
compound compared to all its metabolites.

Metabolism Studies

In Vivo Studies

Studies on in vivo metabolism using whole living animals do not give readily interpretable data as to the kind of processes taking place which affect the fate of the xenobiotics in the body. What we learn from such studies is only the end result. To get a better understanding of the basic mechanism of metabolism and excretion of any given xenobiotic, an in vitro study must accompany the in vivo study.

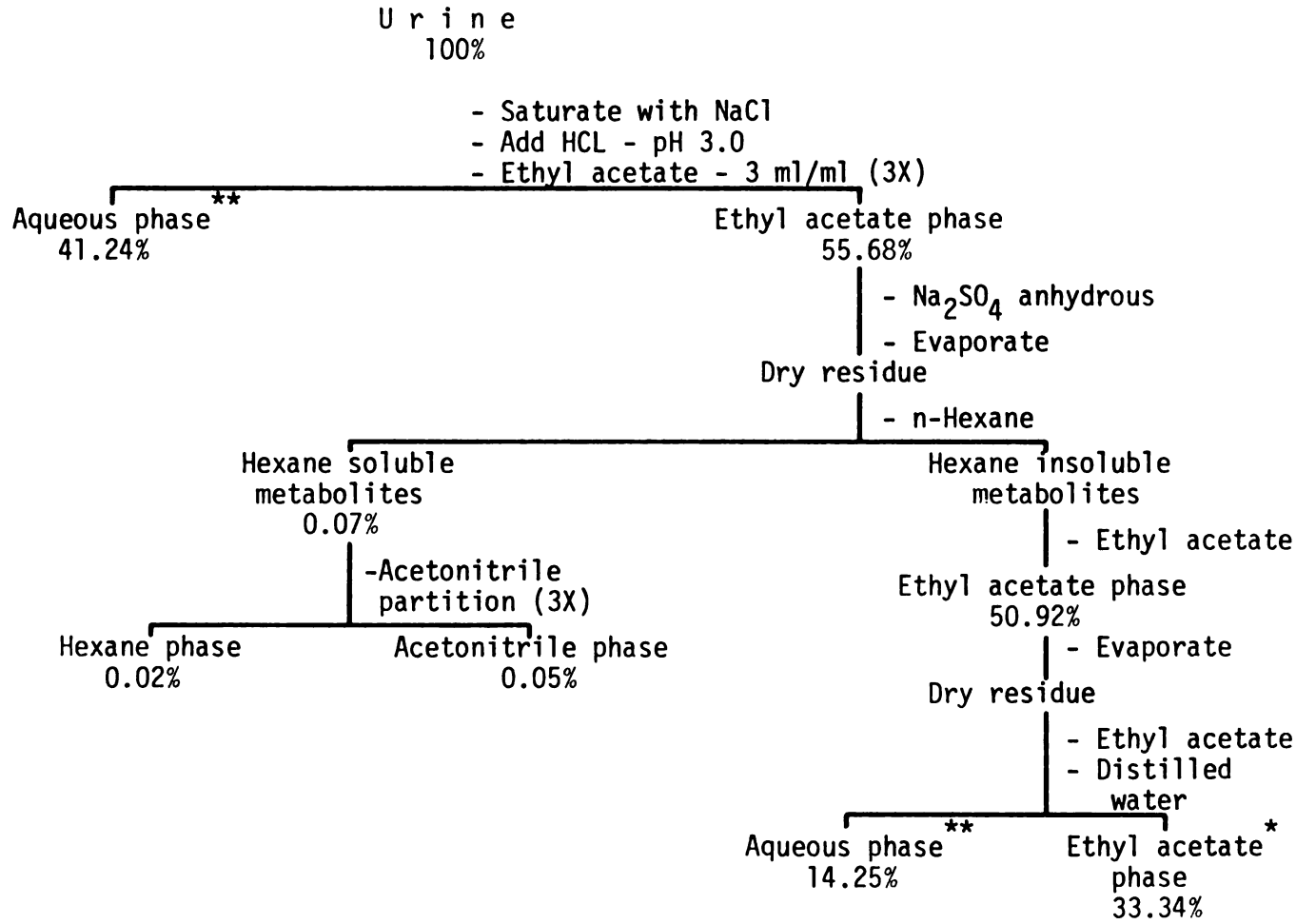
The general scheme of extraction and partition behaviour of metabolites in both feces and urine are shown in Figure 2 and Figure 3. Acetone could extract only about 22% of the radioactivity, while methanol extracted an additional 33%. The total amount of metabolites that can be extracted by solvents was only 55%. By analyzing the methanol extracted solid feces in a biological oxidizer, another 40% of the radioactive metabolite were recovered. This result indicates that in living rats about 40% of the ^{14}C-NK-592 was metabolized into very polar metabolites that were not extractable by solvents or that some metabolites exist in the bound form in the fecal material. Partitioning of the acetone extract, on the other hand, showed that almost equal amounts of metabolites were found in hexane-soluble and hexane-insoluble fractions. It must be noted that almost no radioactivity was found in the aqueous phase. Partitioning of the methanol extract gave about equal amounts of metabolites in ethyl acetate phase as well as aqueous phase.



*These fractions were further analyzed by TLC.
**This fraction was analyzed by deconjugation enzymes and dilute acid.
The figures below each phase represent the percent radioactivity present in that phase as calculated from the initial amount.

The solvent extractable metabolites	55.27%
The solvent inextractable metabolites	40.47%
T o t a l	95.74%

Figure 2. Extraction scheme for the analysis of metabolites in the feces.



*This fraction was analyzed by TLC.

**These fractions were analyzed by deconjugation enzymes and dilute acid.

The figures below each phase represents the percent radioactivity present in that phase as calculated from the initial amount.

Figure 3. Extraction scheme for the analysis of metabolites in the urine.

The ethyl acetate could only extract about 56% of the total metabolites found in the urine leaving behind the very polar water soluble metabolites. Of the 56% solvent extractable metabolites none were found to be hexane-soluble, indicating that most of the metabolites were polar. During partitioning, about 28% of the hexane-insoluble fraction transferred to the aqueous phase, indicating that this portion of the metabolites was indeed polar and truly water soluble.

Analysis of solvent extractable metabolites

The nature of these metabolites in both ethyl acetate fractions from methanol and acetone extracts, and acetonitrile fraction of feces and ethyl acetate fraction of urine were studied on TLC, with a hexane-chloroform-acetonitrile (12:8:11) mobile phase and compared to the ^{14}C-NK-592. Autoradiography of those plates were prepared (Fig. 4 and 5). The amount of radioactivity in each silica gel portion, corresponding to the darkened spots on the autoradiogram were scraped and radioassayed. Tables 9 and 10 show the relative abundance of each metabolite in different portions on the TLC.

The acetonitrile fraction consisted mainly of the starting compound ^{14}C-NK-592 (R_f = 0.76) and one other metabolite which had R_f equal to 0.73. The compound with R_f = 0.73 corresponds to the first oxidation product of NK-592, the sulfoxide ether (Appendix II). On the other hand, the two ethyl acetate fractions of fecal extracts consisted mainly of much more polar metabolites than the one found in the acetonitrile fraction. These metabolites are likely the products of further oxidation reactions on NK-592 and its sulfoxide ether as judged by the degree of polarity of these compounds as compared to the starting material.

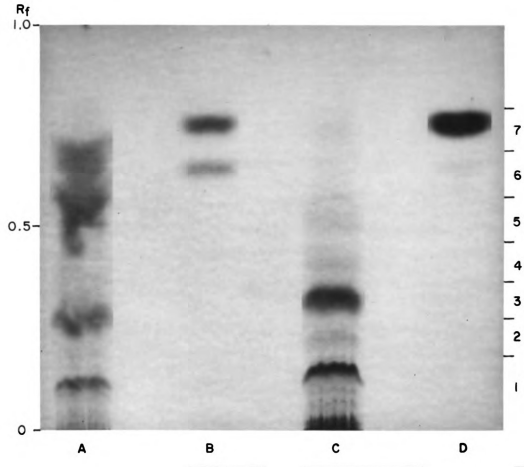


Figure 4. Autoradiogram of thin-layer chromatography of solvent extractable fractions from feces developed in a mixture of hexane-chloroform-acetonitrile (12:8:11) as mobile phase.

A - Ethyl acetate fraction from the acetone phase.
B - Acetonitrile fraction from the acetone phase.
C - Ethyl acetate fraction from the methanol phase.
D - Standard NK-592.

Table 9. Relative Abundance (in percent) of Metabolic Products in Feces Extracts as Analyzed by TLC Shown in Fig. 4.

TLC fraction number[a]	NK-592	Acetone phase		Methanol phase
		Acetonitrile fraction	Ethyl acetate fraction	Ethyl acetate fraction
1	0	2.56	18.14	50.11
2	0	0.46	9.85	6.44
3	0	1.09	11.17	31.79
4	0	0.53	7.41	4.97
5	0	0.69	25.76	3.96
6	0.70	23.81	22.26	1.55
7	99.30	70.86	5.46	1.19

[a]TLC fraction numbers correspond to the metabolite region.

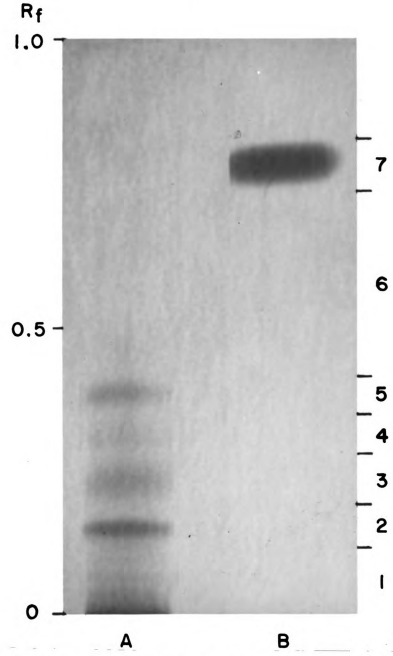


Figure 5. Autoradiogram of thin-layer chromatography of solvent extractable fraction from urine developed in a mixture of hexane-chloroform-acetonitrile (12:8:11) as mobile phase.

A - Ethyl acetate fraction of hexane insoluble phase.
B - Standard NK-592.

Table 10. Relative Abundance (in percent) of Metabolic Products in Urine Extract Analyzed by TLC Shown in Fig. 5.

TLC fraction number[a]	NK-592	Ethyl acetate fraction
1	0	36.09
2	0	23.88
3	0	18.01
4	0	7.78
5	0	9.00
6	0.52	5.18
7	99.48	0.05

[a]TLC fraction numbers correspond to the metabolite region.

The ethyl acetate extract of urine contained mostly metabolites having low R_f values in the same TLC system, indicating that these compounds are polar metabolites. Both feces and urine contained a considerable amounts of metabolites that were more polar than the most polar candidate metabolite available.

To further study the nature of these metabolites, relatively high amounts of each metabolite were required. This was achieved by spotting the extracts on TLC plate as a band, developing the TLC plate phase and preparing an autoradiograph. The silica gel bands containing radioactive metabolites were collected and extracted with acetone. The acetone extracts were respotted side by side with the standard candidate metabolites on TLC plates and developed in different mobile phases (Appendix II). In this way the R_f values of the metabolites were accurately measured. The same clean up method was adopted for acetone extracts, and the resulting partially purified metabolites were analyzed on GLC with an alkali flame ionization detector (Appendix III). In this way some of the metabolites were identified. In feces the major metabolites were NK-592, γ-hydroxy sulfoxide ether and sulfoxide ether. One other minor metabolite that could be positively identified was γ-hydroxy sulfone ether. In addition there were many unknown minor polar compounds having low R_f values. While in urine only γ-hydroxy sulfoxide ether was found.

Analysis of water soluble metabolites

The result of the analysis of the water soluble metabolites is summarized in Table 11. The addition of deconjugation enzymes such as β-glucuronidase and sulfatase did not increase the percent

Table 11. Analysis of Aqueous Fractions from Feces and Urine by Acid Hydrolysis and Deconjugation Enzymes β-Glucuronidase and Sulfatase.

Treatment	Percent radioactivity extracted in diethyl ether		
	Feces	Urine	
	Aqueous phase of hexane-insoluble fraction	Urine after organic solvent extraction	Aqueous phase of hexane-insoluble fraction
Control[a]	11.30 (11.12 - 11.48)	3.99 (3.56 - 4.42)	8.60 (8.31 - 8.87)
β-Glucuronidase	11.28 (10.76 - 11.79)	4.44 (3.62 - 5.26)	7.52 (6.94 - 8.10)
Sulfatase	11.02 (10.29 - 11.74)	6.62 (6.45 - 6.79)	10.38 (9.94 - 10.84)
Acid hydrolysis (HCl)	11.54 (14.51 - 15.97)	15.34 (15.20 - 15.47)	12.01 (11.58 - 12.44)

[a]Aqueous fraction was incubated with acetate buffer pH 5.0 only.

radioactivity that was extractable by ether. Only acid hydrolysis gave some increase in the percent of ether extractable radioactivity. Apparently there were no glucuronide or sulfate conjugate present in the aqueous phase. The water soluble metabolites might be found in forms other than glucuronide and sulfate conjugates.

In vitro Studies

It is generally considered that the increase in the amount of water soluble metabolites in vitro indicates the increase of metabolism of apolar pesticides. This is because all lipid soluble pesticides must be first converted into more water soluble forms before it can be excreted out of the body.

The results of in vitro incubation studies are summarized in Table 12. It is clear from those data that NADPH significantly and consistently increased the conversion of ^{14}C-NK-592 into ether inextractable metabolites. NADPH is a cofactor known to stimulate the mixed-function oxidase which is one of the most important enzymes responsible for the metabolism of xenobiotics. The addition of 0.05 mM SKF-525A (2 x substrate concentration), a known inhibitor of the mixed-function oxidase, to incubation mixtures containing crude supernatant, inhibited the production of water soluble metabolites by 56%. This could mean that there is some endogeneous NADPH or its generating system present in the crude supernatant that stimulates the metabolism of NK-592. The addition of NADPH to the incubation mixture containing crude supernatant increased the production of water soluble metabolites by 326%, whereas the addition of 0.05 mM of SKF-525A reduced the production of water soluble metabolites of

Table 12. Effect of Various Cofactors and Inhibitors[a] Upon the Production of Water Soluble Metabolites *In Vitro* Using 20,000 x g Crude Supernatant.

Treatment	% water soluble metabolites formed from added NK-592[b]	picomole water soluble metabolites formed per mg protein in 2 hr.[b]
Control	2.34 ± 0.33 (3)	120.93 ± 19.20 (3)
Control + SKF-525A	1.04 ± 0.21 (4)*	52.35 ± 10.49 (4)*
Control + GSH	1.13 ± 0.32 (4)	54.40 ± 14.52 (4)
Control + GSH + NEM	0.85 ± 0.36 (4)	41.82 ± 18.06 (4)
Control + GSH + DTT	0.98 ± 0.34 (4)	48.42 ± 17.78 (4)
Control + GSH + PCMB	1.60 ± 0.58 (4)	79.30 ± 29.69 (4)
Control + NADPH	9.96 ± 2.97 (4)**	493.89 ±156.21 (4)**
Control + NADPH + SKF-525A	1.14 ± 0.49 (4)*	65.03 ± 23.99 (4)*
Control + UDPGA	1.34 ± 0.30 (4)	69.15 ± 17.38 (4)
Control + UDPGA + NADPH	16.96 ± 2.48 (4)**	873.53 ±163.82 (4)**
Control + APS + ATP + Mg^{+2}	1.14 ± 0.47 (3)	61.17 ± 43.62 (3)
Control + APS + ATP + Mg^{+2} + NADPH	17.94 ± 6.19 (4)**	944.02 ±357.12 (4)**

[a]Concentrations of GSH, NEM, DTT and PCMB were 4 mM; NADPH, UDPGA, ATP and APS were 2 mM; $MgCl_2$ 10mM and SKF-525 A 0.05 mM (2 x substrate concentration).

[b]Those above data are mean SE of two individual experiments with 2 replications each except treatments Control + SKF-525A and Control + APS + ATP + Mg^{+2}.

*SKF-525A inhibition over either control or NADPH fortified system is significant at $p \leq 0.05$.

**NADPH stimulation over either control, UDPGA fortified or APS + ATP + Mg^{+2} fortified system is significant at $P \leq 0.05$.

the above system by 89%. These data support the notion that these oxidative enzymes are important in the metabolism process of NK-592. These enzymes are known to locate in the microsomal fraction of the liver homogenate and require the presence of both oxygen and NADPH for their activity. On the other hand addition of GSH, a known cofactor for the glutathione-S-transferase did not increase the production of water soluble metabolites. Even the addition of inhibitors such as NEM, DTT and PCMB did not have much influence upon the production of water soluble metabolites. This indicates that metabolism of NK-592 is mainly carried out by mixed-function oxidase rather than glutathione-S-transferase.

After being metabolized by the liver enzymes, some metabolites can further form conjugates such as glucuronide and sulfate conjugates. To form these kinds of conjugates, some other cofactors beside NADPH are needed. Glucuronide conjugate can only be formed in the presence of UDPGA. In this experiment it has been shown that the addition of UDPGA alone did not increase the amount of water soluble metabolites. However, the addition of UDPGA and NADPH did significantly increase (625% over control) the amount of water soluble metabolites. This indicates that some metabolites were able to form conjugates with UDPGA. For sulfate conjugates, the presence of APS, ATP and Mg^{+2} are required. These 3 cofactors react together and form PAPS, the sulfate donor. Here it was found that APS, ATP and Mg^{+2} alone did not increase the amount of water soluble metabolites. However, if NADPH was added to the above system, a significant increase (667% over control) in the amount of water soluble metabolites was observed.

To verify the localization of these metabolic enzymes,

experiments were conducted using both the 100,000 x g microsomal fraction and supernatant. The results are summarized in Table 13. The addition of NADPH to incubation mixtures containing the microsomal fraction or microsomal fraction plus UDPGA did increase the percent water soluble metabolites by 162% and 259% respectively. The addition of SKF-525A to incubation mixtures containing (a) the microsomal fraction, (b) the microsomal fraction plus NADPH and (c) the microsomal fraction plus NADPH and UDPGA inhibited the production of water soluble metabolites by 65%, 40%, and 52% respectively. These experiments prove that mixed-function oxidase enzymes are located in the microsomal fraction of the liver homogenate.

Enzymes responsible for the formation of glutathione conjugates are known to be present in the supernatant of liver homogenate. In these experiments, GSH was not found to enhance the formation of water soluble glutathione conjugates. In the presence of inhibitors of glutathione-S-transferase such as NEM, DTT and PCMB, the amount of water soluble metabolites did not decrease. This indicates that NK-592 and its metabolites were not utilized by the enzyme glutathione-S-transferase.

Analysis of solvent extractable metabolites

Generally speaking, the amount of solvent extractable metabolites was related to the amount of water soluble metabolites produced, which could give an indication of the processes that took place and also the rate of metabolic processes. It is for this reason that *in vitro* metabolism studies were conducted. By studying the nature of solvent extractable metabolites, which are merely intermediates and represent

Table 13. Effect of Various Cofactors and Inhibitors[a] Upon the Production of Water Soluble Metabolites *In Vitro* Using Both 100,000 x g Microsome and Supernatant

Treatment	% water soluble metabolites formed from added NK-952[b]	picomole water soluble metabolites formed per mg protein in 2 hr.[b]
Microsome.		
Control	11.26 ± 1.54 (4)	1,279.31 ± 175.03 (4)
Control + SKF-525A	3.91 ± 0.36 (4)*	444.76 ± 40.97 (4)*
Control + NADPH	29.48 ± 0.91 (4)**	3,348.91 ± 102.60 (4)**
Control + NADPH + SKF-525A	17.71 ± 1.41 (4)*	2,012.58 ± 160.35 (4)*
Control + UDPGA	8.94 ± 1.19 (4)	1,015.52 ± 135.44 (4)
Control + UDPGA + NADPH	40.44 ± 3.57 (4)**	4,593.48 ± 405.72 (4)**
Control + UDPGA + NADPH + SKF-525A	19.33 ± 0.47 (4)*	2,196.13 ± 53.61 (4)*
Supernatant		
Control	1.18 ± 0.31 (4)	140.01 ± 37.28 (4)
Control + GSH	3.09 ± 0.91 (4)	367.02 ± 107.59 (4)
Control + GSH + NEM	2.96 ± 0.57 (4)	351.98 ± 67.88 (4)
Control + GSH + DTT	2.12 ± 0.32 (4)	252.19 ± 37.85 (4)
Control + GSH + PCMB	3.00 ± 0.63 (4)	357.30 ± 74.69 (4)

[a]Concentrations of GSH, NEM, DTT and PCMB were 4 mM; NADPH and UDPGA are 2 mM and SKF-525A was 0.05 mM.

[b]Those above data are mean ± SE of two individual experiments with 2 replications each.

*SKF-525A inhibition over either control or NADPH fortified system is significant at $P \leq 0.05$.

**NADPH stimulation over either control, UDPGA fortified system is significant at $P \leq 0.05$.

unstable stages of metabolism, a better understanding of its metabolic pathway can be obtained. The content of the ether extracts were analyzed by TLC using a mixture of hexane-chloroform-acetonitrile (3:2:2) as the mobile phase. This system gives a reasonably good separation for many of the metabolites. Autoradiography and quantitation of darkened spots were performed as before. Fig. 6 shows the autoradiogram and Table 14 shows the result of the quantitation of the TLC for different extracts. Crude supernatants with or without GSH did not metabolize NK-592. The only metabolite produced under the experimental condition was the sulfoxide ether. No enzymatic reaction was required to produce the sulfoxide ether because NK-592 was not stable and easily oxidized. There are many more polar metabolites formed upon addition of NADPH to the incubation mixture containing crude supernatant. This is likely to be due to the presence of microsomes in the supernatant. About 99% of the parent compound was metabolized into more polar compounds within 2 hours of incubation. Addition of an inhibitor (SKF-525A) did inhibit the metabolism drastically. The amount of parent compound metabolized was only about 70% and much reduced quantities of polar compounds were formed; about 1% compared to 11.5% when no inhibitor was added. When inhibitor was present, the metabolism process proceded only until the sulfoxide and sulfone ether were formed.

The rate of production of solvent extractable metabolites could give the overall rate of metabolic processes. Results of such an experiment are summarized in Fig. 7. Without NADPH the amount of ether extractable metabolites dropped from approximately 89% to about

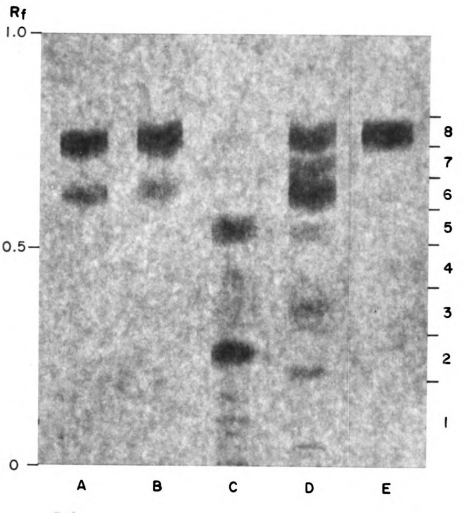


Figure 6. Autoradiogram of thin-layer chromatography of ether extractable metabolites from in vitro studies using 20,000 x g crude supernatant developed in a mixture of hexane-chloroform-acetonitrile (3:2:2) as mobile phase.

A - Ether extract of crude supernatant.
B - Ether extract of crude supernatant + GSH.
C - Ether extract of crude supernatant + NADPH.
D - Ether extract of crude supernatant + NADPH + SKF-525A.
E - Standard NK-592.

Table 14. Relative Abundance (in percent) of the Ether Extractable Metabolites of ^{14}C-NK-592 *In Vitro* as Analyzed by TLC Shown in Fig. 6.

TLC fraction number[a]	NK-592	Crude supernatant only	Crude supernatant + GSH	Crude supernatant + NADPH	Crude supernatant + NADPH + SKF-525A
1	0	0	0	11.47	1.06
2	0	0	0	61.48	3.94
3	0	0	0	4.62	6.60
4	0	0	0	3.12	0.89
5	0	0.42	0.19	17.50	3.67
6	0.37	11.61	6.39	0.40	40.78
7	3.98[b]	20.28[b]	12.08[b]	0.38	13.37
8	95.65[b]	67.70[b]	81.34[b]	1.03	29.70

[a]TLC fraction numbers correspond to the metabolite regions.

[b]Those two fractions are actually one compound, i.e. NK-592.

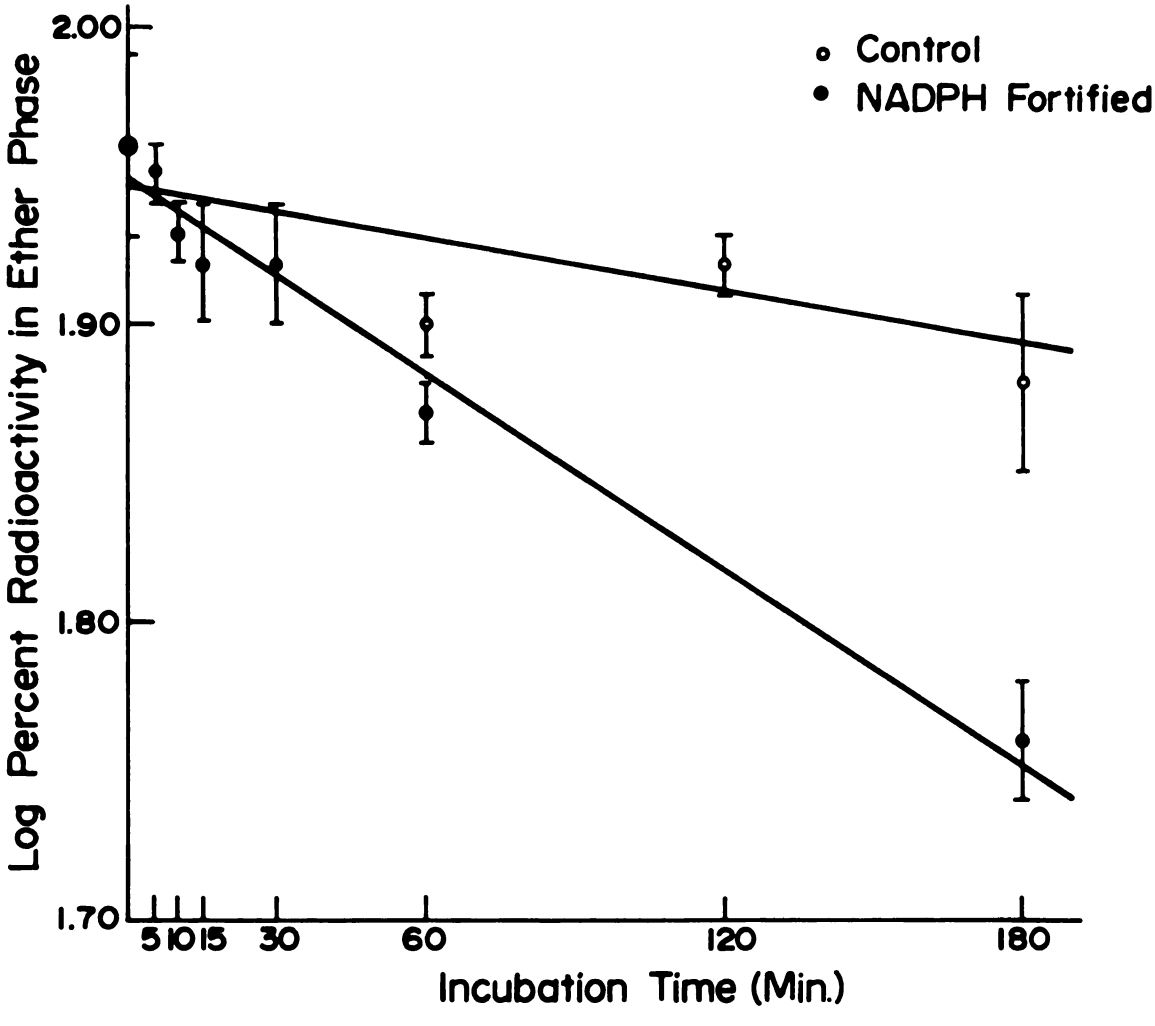


Fig. 7. Percentages of ether extractable radioactivity (expressed in a logarithmic scale) of mixtures incubated for different time periods.

79%. However, in the presence of NADPH it dropped to approximately 56% within 3 hours of incubation. The content of the ether extracts were once again analyzed by TLC using the same solvent mixture as the mobile phase. Fig. 8 shows the autoradiogram of ether extracts of the reaction mixtures incubated at different times from 5 min to 180 min. Table 15 shows the relative abundance of metabolites present in the ether extracts. From these results a metabolic sequence can be predicted. NADPH strongly activated the metabolic processes as judged from the previous experiments and the number of intermediates produced. During the oxidation process, sulfoxide ether was formed as the first metabolite which was then further oxidized to sulfone ether and eventually to more polar compounds such as alcohols. Less polar compounds (NK-592, sulfoxide ether and sulfone ether) started to disappear after 30 min. of post-incubation, while polar metabolites ($R_f < 0.28$) started to appear within 15 min. As a result of TLC and GLC analysis, the following metabolites were positively identified; in the ether extract of the NADPH fortified incubation mixture γ-hydroxy sulfoxide ether and β-hydroxy sulfone ether were the two major metabolic products. Other significant metabolites were sulfoxide ether, β-hydroxy sulfoxide ether, γ-hydroxy sulfone ether and sulfone ether. Five unidentified minor metabolites were recognized; they were very polar and the total amount was approximately 10%.

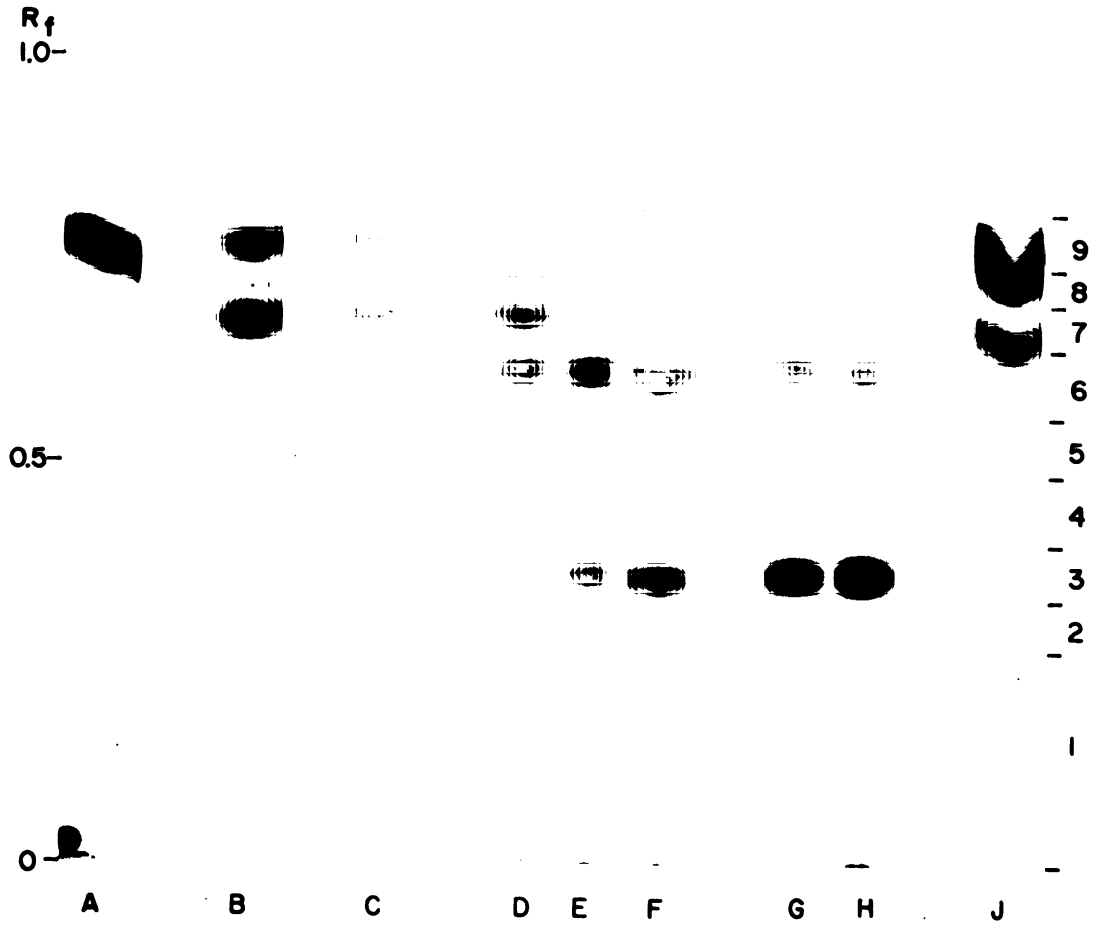


Figure 8. Autoradiogram of thin-layer chromatography of ether extractable metabolites from in vitro studies using 20,000 x g crude supernatant incubated for different time period and developed in a mixture of hexane-chloroform-acetonitrile (3:2:2) as mobile phase.

A - Standard NK-592.
B to H - Ether extracts of crude supernatant + NADPH incubated for 5, 10, 15, 30, 60, 120 and 180 min.
J - Ether extract of crude supernatant incubated for 120 min.

Table 15. Relative Abundance (in percent) of the Ether Extractable Metabolites of ^{14}C-NK-592 *In Vitro* as Analyzed by TLC Shown in Fig. 8.

TLC fraction number[a]	NK-592	Crude supernatant without NADPH[b]	Crude supernatant with NADPH						
			Incubation time (min)						
			5	10	15	30	60	120	180
1		0.20	0.77	1.95	2.65	5.72	7.76	9.33	9.11
2		0.07	0.53	0.92	1.17	1.59	2.17	1.71	1.19
3		0.30	0.88	2.88	6.12	20.38	40.86	58.35	63.88
4		0.17	6.34	9.12	11.42	12.77	8.80	5.16	3.94
5		0.30	0.80	1.56	3.15	4.02	3.72	3.97	5.30
6		4.60	6.75	14.12	22.62	35.08	30.37	17.91	13.15
7	1.93	19.22	45.52	29.90	26.09	10.93	1.90	2.92	2.06
8	12.81[c]	34.07	12.05	16.35	17.15	7.92	1.53	0.65	0.34
9	85.26[c]	41.08	26.37	23.27	9.64	1.60	2.89	0.68	1.02

[a]TLC fraction numbers correspond to the metabolite regions shown in Fig. 5.

[b]This system was incubated for 120 minutes.

[c]Fractions 8 and 9 of NK-592 is not clearly separated.

Analysis of Water Soluble Metabolites

The result of the analysis of the water soluble metabolites is summarized in Table 16. During the incubation of crude supernatant with NK-592, NADPH significantly increased the amount of water soluble metabolites. However, deconjugation and acid hydrolysis of water soluble metabolites from the aqueous phase of NADPH fortified crude supernatant produced less of the ether extractable metabolites as compared to the aqueous phase of crude supernatant only. Therefore, the increase of water soluble metabolites upon addition of NADPH was not due to the increase in glucuronide and sulfate conjugates. It might be due to the increase of forms other than those two types of conjugates. This is in accordance with the results obtained from similar experiments done on the aqueous phase of both feces and urine.

Mutagenic Effect

The purpose of this study was to examine the potential of the candidate metabolites to cause mutation by using the Ames test. The positivity in the Ames test does not automatically indicate the compound is mutagenic and carcinogenic *in vivo*. The first thing to consider is whether the mutagenic metabolite is found *in vivo* in a sufficiently large quantity. The second consideration is the presence of groups of compounds which give either false negative or false positive as compared to *in vivo* carcinogenicity-mutagenicity test results. Some compounds such as chlorinated hydrocarbon insecticides which cause cancer in laboratory animals *in vivo* for instance do not show up as mutagens in the Ames test. However, the Ames test serves

Table 16. Analysis of Aqueous Fraction from In Vitro Studies by Acid Hydrolysis and Deconjugation Enzymes β-glucuronidase and sulfatase.

Treatment	Percent radioactivity extracted in diethyl ether	
	Crude supernatant	Crude supernatant + NADPH
Control[a]	68.86 (67.70 - 70.02)	15.80 (15.75 - 15.85)
β-Glucuronidase	60.93 (54.37 - 67.49)	17.82 (17.50 - 18.14)
Sulfatase	52.78 (51.62 - 53.94)	15.52 (15.19 - 15.85)
Acid hydrolysis (HCl)	74.04 (72.56 - 75.53)	32.38 (30.69 - 34.07)

[a]Aqueous fraction was incubated with acetate buffer only at pH 5.0.

as a quick preliminary test which may be used to judge the potential hazard in this regard, and is particularly suited for newly developed and field untested compounds.

The results shown in Table 17 indicate that the parent compound NK-592 has a very slight mutagenic activity with or without metabolic activation by the S_9 mix. The two immediate oxidative products (sulfoxide ether and sulfone ether) gave a little higher activity than NK-592. Further experiments were carried out to examine the effect of concentration changes and the result are shown in Table 18. Here again NK-592 itself did not show a significant degree of mutagenicity. Instead it exhibited some degree of cytotoxicity against the bacteria at high concentrations. The sulfoxide metabolite on the other hand did show a dose-related response effect, both with and without metabolic activation. The mutagenicity effect was significant even at the lowest concentration (i.e. 25 μg/plate), and the degree of mutagenicity at high concentrations was quite apparent (i.e. 1,000 μg/plate or more). On the other hand the sulfone metabolite did not show as high a degree of mutagenic effect, though it is more mutagenic than NK-952. Table 19 shows the results of the mutagenicity test of other candidate metabolites. Certain of these showed some degree of mutagenicity with or without metabolic activation. All hydroxylated metabolites, whether it is at the β or γ position of the thiopropyl side chain, are mutagenic to varying extents. The sulfoxides and hydroxylated products were the metabolites that were found in vivo.

Table 17. Mutagenicity Test of NK-592 and Some of Its Candidate Metabolites Using Ames Bacterial System Salmonella typhimurium Strain TA 100; the Data are Expressed in the Number of Revertant (Mutation) Colonies Observed Per Plate

Treatment[a]		-S_9 mix[c]	+ S_9 mix[c]
DMSO		105	118
NQNO[b]	5 µg	> 1,000	> 1,000
NK-592	1,000 µg	123	178
Sulfoxide ether	1,000 µg	165	216
Sulfone ether	1,000 µg	155	184
Sulfide phenol	1,000 µg	118	130
Sulfoxide phenol	1,000 µg	122	116
Sulfone phenol	1,000 µg	0	0

[a]Amount of mutagen used is expressed in terms of µg/plate.

[b]NQNO = 4-Nitroquinoline-N-oxide, a known mutagen used as positive control.

[c]S_9 mix = liver homogenate centrifuged at 9,000 x g for 10 min., suspended in 0.2 M sodium phosphate buffer pH 7.4 plus KCl, $MgCl_2$, NADP and glucose-6-phosphate.

Table 18. Mutagenicity Test of NK-592; Sulfoxide Ether and Sulfone Ether at Different Concentrations Using Ames Bacterial System Salmonella typhimurium Strain TA 100; the Data are Expressed in the Number of Revertant (Mutation) Colonies Observed Per Plate

Treatment[a]		$-S_9$ mix[c]	$+S_9$ mix[c]
DMSO		157	170
NQNO[b]	5 µg	> 1,000	> 1,000
NK-592	250 µg	201	298
NK-592	500 µg	177	311
NK-592	1,000 µg	169	262
NK-592	2,000 µg	145	263
Sulfoxide ether	25 µg	194	258
Sulfoxide ether	50 µg	231	256
Sulfoxide ether	100 µg	337	424
Sulfoxide ether	200 µg	297	410
Sulfoxide ether	500 µg	470	583
Sulfoxide ether	1,000 µg	867	798
Sulfoxide ether	1,500 µg	1,090	972
Sulfone ether	25 µg	256	323
Sulfone ether	50 µg	294	328
Sulfone ether	100 µg	361	371
Sulfone ether	200 µg	352	445
Sulfone ether	500 µg	384	500
Sulfone ether	1,000 µg	341	433
Sulfone ether	1,500 µg	334	413

[a]Amount of mutagen is expressed in terms of µg/plate.

[b]NQNO = 4-Nitroquinoline-N-oxide, a known mutagen used as positive control.

[c]S_9mix = liver homogenate centrifuged at 9,000 x g for 10 min, suspended in 0.2 M sodium phosphate buffer pH 7.4 plus KCl, $MgCl_2$, NADP and glucose-6-phosphate.

Table 19. Mutagenicity Test of Candidate Metabolites of NK-592 at Different Concentrations Using Ames Bacterial System Salmonella typhimurium Strain TA 100; the Data are Expressed in the Number of Revertant (Mutation) Colonies Observed Per Plate

Treatment[a]		-S_9 mix[b]	+S_9 mix[b]
DMSO		110	116
Benzo(a)pyrene	10 µg	108	318
Aminoanthracene	10 µg	123	1,012
γ-Hydroxy sulfide ether	100 µg	138	179
γ-Hydroxy sulfide ether	500 µg	220	344
γ-Hydroxy sulfoxide ether	100 µg	227	202
γ-Hydroxy sulfoxide ether	500 µg	746	611
γ-Hydroxy sulfone ether	100 µg	192	184
γ-Hydroxy sulfone ether	500 µg	528	678
β-Hydroxy sulfide ether	100 µg	154	238
β-Hydroxy sulfide ether	500 µg	229	445
β-Hydroxy sulfoxide ether	100 µg	177	198
β-Hydroxy sulfoxide ether	500 µg	507	509
β-Hydroxy sulfone ether	100 µg	152	184
β-Hydroxy sulfone ether	500 µg	394	487
Amino sulfide ether	100 µg	112	154
Amino sulfide either	500 µg	47	105
Amino sulfoxide ether	100 µg	114	173
Amino sulfoxide ether	500 µg	99	224
Amino sulfone ether	100 µg	116	252
Amino sulfone ether	500 µg	106	338
Diphenyl ether	100 µg	> 1,000	> 1,000
Diphenyl ether	500 µg	> 1,000	> 1,000

[a]Amount of mutagen used is expressed in terms of µg/plate.

[b]S_9 mix = liver homogenate centrifuged at 9,000 x g for 10 min, suspended in 0.2 M sodium phosphate buffer pH 7.4 plus KCl, $MgCl_2$, NADP and glucose-6-phosphate.

DISCUSSIONS AND CONCLUSIONS

The toxic symptoms of NK-592 affected mites showed that it is a neuroactive agent; however, because no convulsions were observed, it is not possible to conclude whether NK-592 is a poison that acts on the central nervous system or not. There was no sign of dessication of the dead mites which could mean that NK-592 is not a respiratory poison.

Dicofol, which differs from DDT only in the substitution of a hydroxyl group for the α-hydrogen, is known to be a very effective acaricide. Other chemicals that are known to have good acaricidal activities are chlorobenzilate, sulphenone, DMC, tetradifon, chlorbenside, tetrasul, etc. As a result, it was suggested that acaricidal activity was dependent upon two phenyl groups linked by a suitable bridging structure, phenyl-X-phenyl, and that like DDT, toxicity was maximal when the phenyl groups were p,p'-chloro substituted. Bridging structures which conform to these conditions include: $-OCH_2O-$, $-\overset{\overset{O}{\|}}{S}-O-$, $-CH_2O-$, $-\overset{\overset{O}{\|}}{C}-O-$, $-S-$, $-O-$, $-CH_2S-$ etc. (26). This seems to fit the chemical structure of NK-592, which consists of two phenyl rings that are para,para' substituted and bridged by -O- bond, to form a diphenyl ether compound. Holan (1969, 1971) deduced that all active compounds, including some other DDT analogs, resembled a "molecular wedge." In DDT the apex is the $-CCl_3$ moiety and the base is the two phenyl rings and these must contain electron donating substituents.

Gunther et al. (1954) suggested that DDT bound to a "protein like substance - presumably an enzyme." Kato et al. (1975) suggested that mode of action against adult mites by 4-(Y)-3-(alkylthio) phenyl-4-nitrophenyl ethers and their sulfoxides involves the fitting of the 4-substituent Y on a specified site and the hydrophobic binding of the alkyl radical on a lipoproteinic site. The same explanation can be given for its action against eggs. Matsumura (1976) suggested that lipid solubility seems to be a basic requirement for the insecticidal activity of an insecticide; whereas chemicals that possess good acaricidal activity such as dicofol, chlorobenzilate, sulphenone etc. do not have good insecticidal activity probably because of their higher polarity compared to insecticides, which seems to be a requirement for good acaricides. The parent compound NK-592 also is relatively polar. It is noteworthy that NK-592 is more toxic against two-spotted spider mites compared to its first oxidative product, sulfoxide ether. This is in contrast with the results of Kato et al. (1975), who found that sulfides and sulfoxides of NK-592 exhibited the same order of activity against T. urticae. It has been found that azinphos methyl resistant strain mites are still susceptible to NK-592. This must mean that the resistance mechanism that is effective to azinphos methyl is not useful in protecting the mites from the attack by NK-592. In this connection it is interesting to note that a cross-resistance to Benzomate (Citrazon = ethyl O-benzoyl 3-chloro-2, 6-dimethoxybenzohydroximate) (see Appendix IV) and NK-592 has been reported (Ishida, S., personal communication, 1980). The structure similarities between NK-592 and

Citrazon is not apparent. However, the presence of cross-resistance must mean that some increased defense mechanism employed by the resistant mites work on both compounds.

A proposed metabolic pathway for NK-592 in rats is shown in Fig. 9. The sulfoxidation reaction and hydroxylation at the γ-position of the n-propyl side chain are the major reactions. To understand the significance of such a metabolic pattern one must look into various metabolic reactions that have been reported to occur on diphenyl ether compounds. Shimabukuro et al. (1979) found that hydrolysis of diclofop-methyl to diclofop was the initial degradation reaction and aryl hydroxylation and phenol conjugation were predominant in the detoxification process of diclofop-methyl in wheat. No ether bond cleavage was observed in the case of diclofop. Hunt et al. (1977), found that in sheep 11% of total administered ^{14}C-nitrofen was excreted in feces. Conjugates of amino-nitrofen, 5-hydroxy ether and small amount of phenol were found in urine. Ether bond cleavage was a minor pathway. Gutenmann and Lisk (1967) studied nitrofen metabolism in dairy cows and found no nitrofen residue in milk, urine and feces when fed at 5 ppm for four days. The major metabolite found was amino-nitrofen which was thought to be formed by nitro reduction in the rumen fluid. No conjugates were found after hydrolysis. In rape, redroot pigweed and green foxtail Hawton and Stobbe (1971) found that a small amount of metabolite might be formed by ether bond cleavage of nitrofen. In all those studies it is clear that ether bond cleavage is not a major degradation reaction. This fact is in accordance to Williams (1959) statement regarding the biological stability

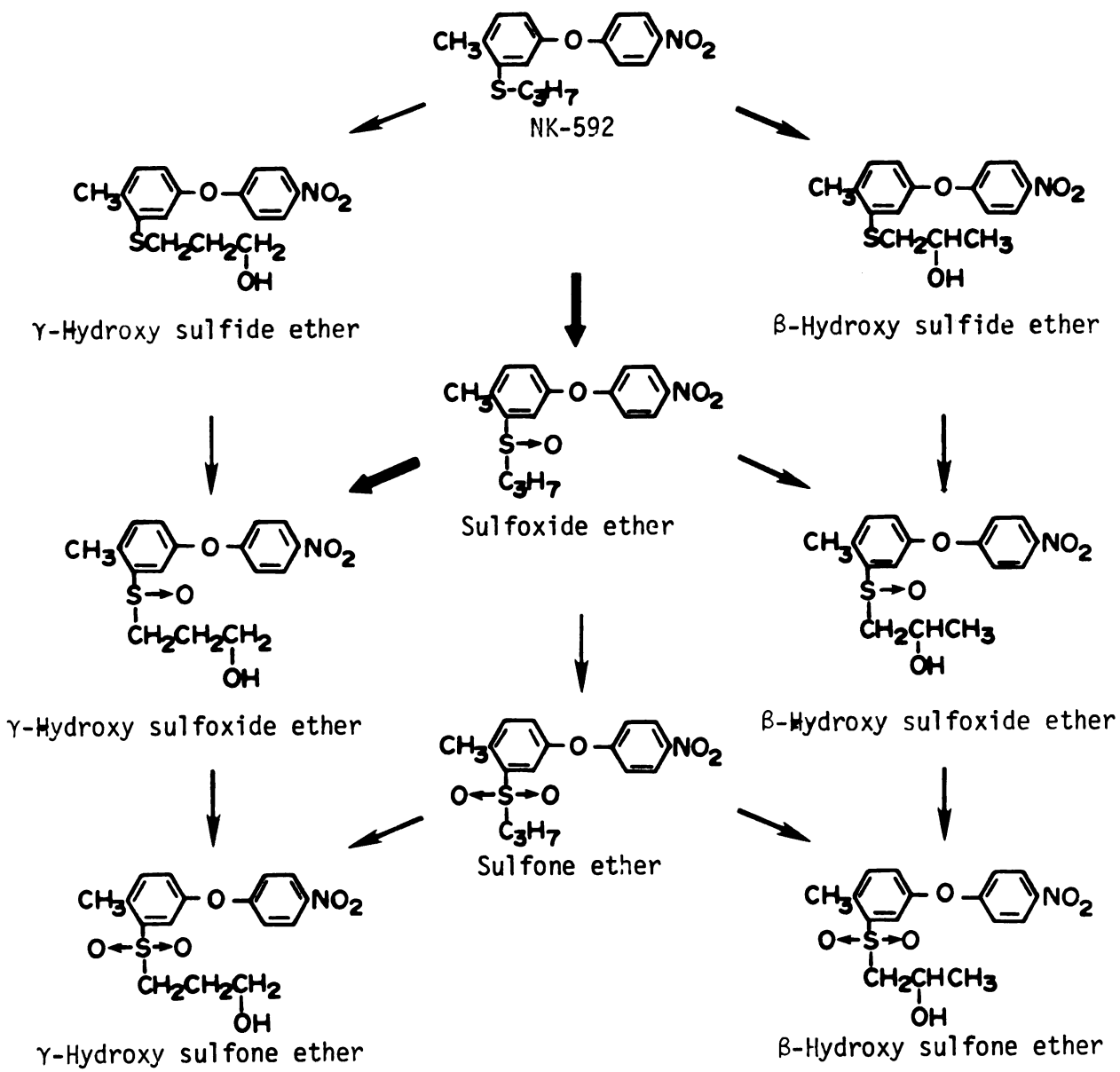


Fig. 9. Proposed metabolic pathway of NK-592 in rat.

of diphenyl ethers. Fluorodifen seems to be an exception, since it is rapidly cleaved at this position. For instance Geissbuchler et al. (1972) found that hydrolysis of the diphenyl ether linkage of fluorodifen to be the major pathway of degradation resulting in the p-nitrophenyl moiety and formation of conjugates with β-D-glucoside in soybean and corn plants. Lamoureux and Davison (1975) found that the major metabolite of fluorodifen found in rat urine was 2-nitro-4-trifluoro-methylphenyl mercapturic acid which accounted for 41% of the dose administered. This means that in rats the mercapturic acid pathway is very important in detoxification. Williams (1959) found that 4-hydroxy diphenyl ether which was excreted mainly as a glucuronide was the principal metabolite of diphenyl ethers fed to rabbits. In this case there is no ether bond cleavage. Rogers (1971) concluded that limited reduction of the nitro substituents and rapid cleavage of the ether linkage to form the corresponding phenol derivatives were the major reactions of fluorodifen in soybean plants. In peanuts Shimabukuro et al. (1973) found that metabolism of fluorodifen catalyzed by glutathione-S-transferase is the most important reaction. That enzyme catalyzes the cleavage of fluorodifen and form conjugates. The reason why no ether bond cleavage takes place in the case of NK-592 could be that the ortho-substitution in this case is not strongly electron withdrawing. It is also interesting to note that no metabolites were detected after hydrolysis of the aqueous phase of urine, feces and from in vitro studies. It must mean that there was no conjugates present in those fractions. In vitro studies, however, indicated that some of the metabolites are capable of forming conjugates as judged by the increase in water

soluble metabolites as a result of incubation with UDPGA or APS (plus ATP and $MgCl_2$) in the presence of NADPH. Therefore it is rather surprising that no glucuronide or sulfate conjugates were recognized *in vivo*. Two reasons could be postulated for this discrepancy. First, the *in vitro* studies may not necessarily represent the processes that take place *in vivo*. Second, there is a chance that the conjugates which were formed in the liver system were enzymatically cleaved during the excretion process in the intestine or in the blood so that no conjugates were recognized either in urine or feces. Furthermore the addition of reduced glutathione, a known cofactor for the formation of glutathione conjugates, did not increase the formation of water soluble metabolites. It means that NK-592 and its metabolites were not preferred substrate for the enzyme glutathione-S-transferase. It must be stressed here that NK-592 is the only diphenyl ether pesticide which has not been shown to go through any kind of conjugation reactions *in vivo*. NK-592 is also unique in that it is not ring hydroxylated despite the fact that it is metabolized by the mixed-function oxidase system. From *in vitro* studies γ-hydroxy sulfoxide ether and β-hydroxy sulfone ether were recognized as major metabolites, whereas sulfoxide ether, β-hydroxy sulfoxide ether, γ-hydroxy sulfone ether and sulfone ether were minor ones. In feces the major compounds detected were NK-592, γ-hydroxy sulfoxide ether and sulfoxide ether plus γ-hydroxy sulfone ether as a minor one; while in urine γ-hydroxy sulfoxide ether was the only one that was recognized.

Upon analysis of body fat for its residue, it was found that

NK-592 was the only compound stored in the fat tissue. This is understandable because NK-592 is more apolar than its metabolites.

NK-592 by itself showed only a slight degree of mutagenicity with or without activation. But its sulfoxide and alcohol metabolites showed somewhat stronger mutagenic activity without metabolic activation. This means that these compounds by themselves have some potency to cause mutation that under certain circumstances could lead to carcinogenicity. This does not mean that this compound and its metabolites are carcinogenic in vivo; it all depends upon how much of these compounds are going to be formed and come in contact with cells of certain organs. This type of test can only give some indications about the carcinogenic potential of certain compounds; further in vivo testing ought to be carried out before any final conclusion can be drawn. However, the value of this type of in vitro testings cannot be overlooked. They are inexpensive, quick and easy to perform and thereby give scientists a quick signal as to the compounds' potential problems and future outlooks of their marketing possibilities.

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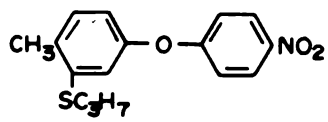
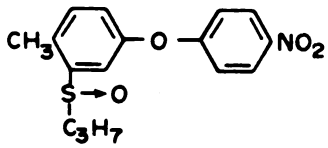
APPENDICES

APPENDIX I

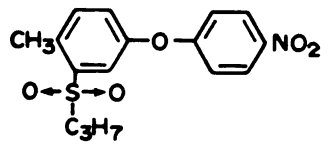
CHEMICAL STRUCTURE OF NK-592 AND ITS METABOLITES



NK-592



Sulfoxide ether



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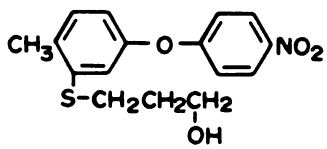
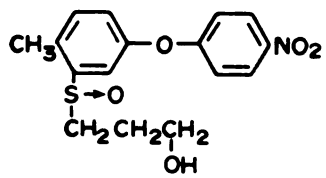
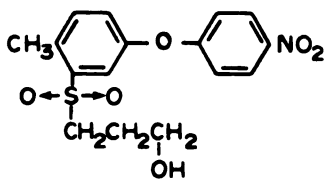


γ-Hydroxy sulfide ether



γ-Hydroxy sulfoxide ether



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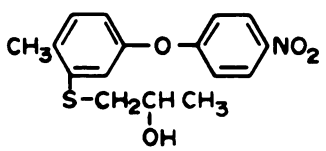
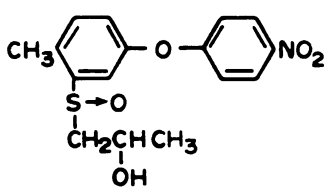
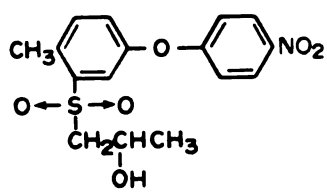


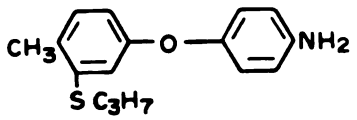
β-Hydroxy sulfide ether



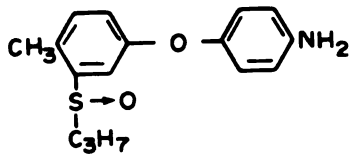
β-Hydroxy sulfoxide ether



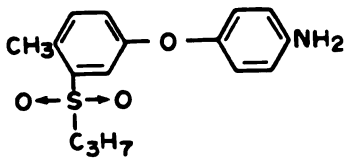
β-Hydroxy sulfone ether



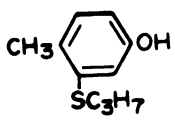
Amino sulfide ether



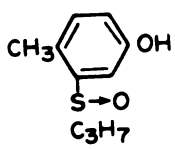
Amino sulfoxide ether



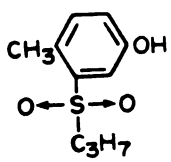
Amino sulfone ether



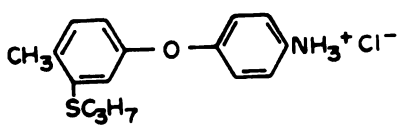
Sulfide phenol



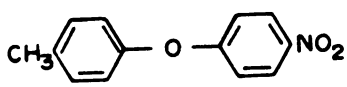
Sulfoxide phenol



Sulfone phenol



Amino chloride sulfide ether



Diphenyl ether

APPENDIX II

R_f VALUES[a] OF DIFFERENT METABOLITES IN DIFFERENT TLC SYSTEM

Chemicals	TLC system[b]				
	I	II	III	IV	V
NK-592	0.77	0.76	0.66	0.87	0.68
Sulfoxide ether	0.67	0.65	0.46	0.72	0.60
Sulfone ether	0.72	0.73	0.54	0.76	0.66
γ-Hydroxy sulfide	0.66	0.66	0.46	0.62	0.52
γ-Hydroxy sulfoxide	0.28	0.26	0.10	0.38	0.15
γ-Hydroxy sulfone	0.47	0.51	0.23	0.43	0.30
β-Hydroxy sulfide	0.69	0.71	0.52	0.67	0.60
β-Hydroxy sulfoxide	0.38	0.38	0.20	0.49	0.23
β-Hydroxy sulfone	0.58	0.63	0.37	0.56	0.48

[a]In all cases the stationary phase was a thin layer (0.25 mm) of silica gel.

[b]Solvent systems that were used as mobile phase were:
I = 3 Hexane - 2 Chloroform - 2 Acetonitrile
II = 12 Hexane - 8 Chloroform - 11 Acetonitrile
III = 2 Hexane - 1 Ether - 1 Acetone
IV = 4 Hexane - 5 Chloroform - 1 Methanol
V = 9 Chloroform - 1 Acetone

APPENDIX III

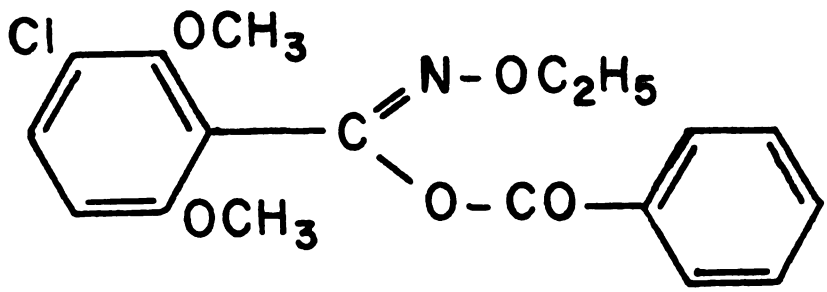
RETENTION TIMES (t_R) OF KNOWN STANDARD METABOLITES IN THREE DIFFERENT GLC SYSTEMS

Chemicals	Retention time[a]						
	3% SE-30			3% OV101		3% QF-1	
	210°	230°	250°	210°	250°	210°	230°
NK-592	6.9	3.1		6.5		5.2	
Sulfoxide ether	1.4			1.4			1.4
Sulfone ether		6.8		15.4			19.1
γ-Hydroxy sulfoxide ether	1.5			1.4		1.3	
γ-Hydroxy sulfone ether + TMS[b]			7.5		6.9		
β-Hydroxy sulfoxide ether	1.4			1.4		1.3	
β-Hydroxy sulfone ether		5.3					14.9

[a]Retention time is expressed in minutes.

[b]γ-Hydroxy sulfone ether + TMS = a trimethyl silyl derivative of the compound γ-hydroxy sulfone ether.

APPENDIX IV



BENZOMATE
(CITRAZON)