Metabolic Fate of Ethylenethiourea in Pregnant Rats

by Y. Kato, Y. Odanaka, S. Teramoto, and O. Matano

The Institute of Environmental Toxicology
Suzuki-cho 2-772, Kodaira-shi, Tokyo

Ethylenethiourea (2-imidazolidinethione, ETU) is produced from a group of fungicides known as ethylenebisdithiocarbamates such as zineb and maneb by oxigen-dependent degradation (HYLIN, 1973), heat decomposition in the process of cooking (NEWSOME and LAVER, 1973), and metabolic process in animal body (FAO/WHO, 1968). It shows remarkable biological activities and has been reported to be antithyroid (GRAHAM and HANSEN, 1972), carcinogenic (INNES et al., 1969; ULLAND et al., 1972), mutagenic (SEILER, 1974), and teratogenic (KJERA, 1973; TERAMOTO et al., 1975).

Although the biological activities of ETU have been well documented, the mode of action is not well known because of the limited informations of its metabolic fate in animals. Especially in connection to the teratogenic property no information is reported on the metabolism in pregnant animals, and the most basic problem whether ETU is transported to the developing embryos is still remained unsettled. In this experiment metabolic fate of ETU was investigated using C^{14}-ETUs in pregnant rats.

MATERIALS AND METHODS

LABELED COMPOUNDS. 2-C^{14}-ETU and 4,5-C^{14}-ETU were synthesized from C^{14}-carbon disulfide and 1,2-C^{14}-ethylenediamine dihydrochloride respectively according to the method of ALLEN et al., (1967). Their radiochemical purities were found to be more than 98% by thin layer chromatography in either case.

ANIMALS. Females of Wistar Imamichi rats of 12-13 weeks of age were mated overnight with adult males. The day when the vaginal plug was found was designed as day 0 of pregnancy. On the twelfth day of gestation, the rats were administered 100 mg/kg of C^{14}-ETU (45.87 uCi of 2-C^{14}-ETU or 30.45 uCi of 4,5-C^{14}-ETU) by intragastric intubation, which is the dosage enable to induce congenital malformations (TERAMOTO et al., 1975). They were housed individually in glass metabolic cages fitted for the collection of respiratory carbon dioxide and given fresh water and normal diet *ad libitum*.

COLLECTION OF EXPIRED AIR. Expired air drawn from the metabolic cage was dehydrated with phosphorous pentoxide and then trapped in monoethanol amine solution.

Bulletin of Environmental Contamination & Toxicology, Vol. 16, No. 5 © November 1976 by Springer-Verlag New York Inc.

COLLECTION OF TISSUES. Animals were anesthetized with ether and the blood sample was taken from vena cana candalis. Then the whole body was perfused with the physiological saline and the following tissues were collected:brain,pypophysis,thyroid gland,thymus gland,lung,liver,adrenal body,kidney,spleen,muscle,bone marrow, placenta(amnion,fetal placenta,and allantois),amniotic fluid,and fetus.

ANALYSES OF RADIOACTIVITY. A half litter of the fetuses,brain, lung,liver,thymus gland,kidney,spleen,muscle,and placenta were homogenized with five volumes of ethanol-water mixture(1:1,v/v). Aliquotes of the homogenate were digested with NCS tissue solubilizer(Amerscham & Searl) according to the method of common use followed by dissolution in PCS scintillation liquid(Amerscham & Searl). The residue of the homogenate of the fetus or the mixture of serum and ethanol(2:1,v/v) was mixed with a quarter volume of chloroform and centrifuged to be separated into three fractions (ethanol-water,chloroform,and crude protein fraction). The pellet (crude proteins) was washed with 2 ml of ethanol-water(1:1,v/v) and 3 mls of ethanol and acetone three times respectively and digested with NCS. Each of the fetuses of the remaining half litters,thyroid gland,hypophysis,bone marrow,and amniotic fluid were digested immediately with NCS. For the analysis of expired air sample,2 ml of the ethanolamine solution which absorved the expired gas was mixed with 3 ml of ethyleneglycol monomethylether and PCS scintillation liquid. Urine was diluted with water and aliquotes of it were dissolved in PCS. Feces and the clot were combused by Tritium Carbon Oxidizer(Packard TriCarb 306) prior to liquid scintillation counting. Radioactivities were estimated by external channel ratio standerization method using Beckman LC-355 liquid scintillation counter.

THIN LAYER CHROMATOGRAPHY. The ethanol-water fraction of the fetus extract was concentrated at 30°C under nitrogen flow and chromatographed on silicagel thin layer plates(merck 60 F_{254}) in ethylacetate-methanol-25% ammonia water(15:1:1,v/v). The chromatograms were visualized under ultraviolet light(253.6 nm) as well as applying Ehrlich's reagent. For the detection of radioactive metabolites the plate was sectioned into about 30 bands followed by the radiocarbon assay by liquid scintillation counting.

GASCHROMATOGRAPHY. The ethanol-water fraction of the fetus extract was refluxed with benzylchloride as reported by NEWSOME(1972),and the extracted benzylated compounds were introduced to Shimazu GC-4BM gaschromatograph equipped with flamephotometric detecter(filter;394 nm for S atom). Glass column,0.3 cm in inner diameter and 2 m long,was packed with 5% XE-60 on gaschrome Q (60-80 mesh). The column temperature was 180°C,that of the injection port and detecter was 250°C. The flow rate of carrier gas (nitrogen) was 110 ml/min and those of hydrogen and air were 50 and 110 ml/min respectively.

RESULTS

ABSORPTION AND ELIMINATION. Hydrophilic ETU was so readily absorbed from rat gastrointestinal tract that as early as in 5 min after the dosage significant radioactivity appeared in the maternal blood (fig.1). The blood level increased rapidly and reached maximal in 2 hr (0.48 umole as 2-C^{14}-ETU/ g blood) and then decreased to 0.04 umole as 2-C^{14}-ETU / g blood by 24 hr.

The major elimination route of ETU was the urinary one and 12% of the administered activity(4,5-C^{14}-ETU) were eliminated by 3 hr,and the cumultative percent increased to 80.2 ± 3.2% by 24 hr and 82.5 ± 3.1% by two days. In contrast to the ready elimination to the urine,fecal elimination was very low,which was estimated as only 0.53% in two days(fig.2).

EXPIRATION OF RADIOACTIVE CARBON DIOXIDE. When 4,5-C^{14}-ETU was administered the expiration of radioactive air was detected 15 min after the dosage,then the elimination rate(expired activity per 2 min) increased rapidly followed by the decline through three phases till the end of this experimental term,192 hr. The radioactive compound trapped in monoethanol amine was identified as carbon dioxide by making a radioactive white precipitate in barium hydroxide solution. No expiration of radioactive gas was observed either from 4,5-C^{14}-ETU itself,radioactive urine,or feces. In contrast to 4,5-C^{14}-ETU,in the case of 2-C^{14}-ETU only a trace level of the expiration was found (fig.3). These results indicated that the fragmentation of imidazolidine ring of ETU and the decarboxylation of 4 and/ or 5 carbon atoms of ETU occured.

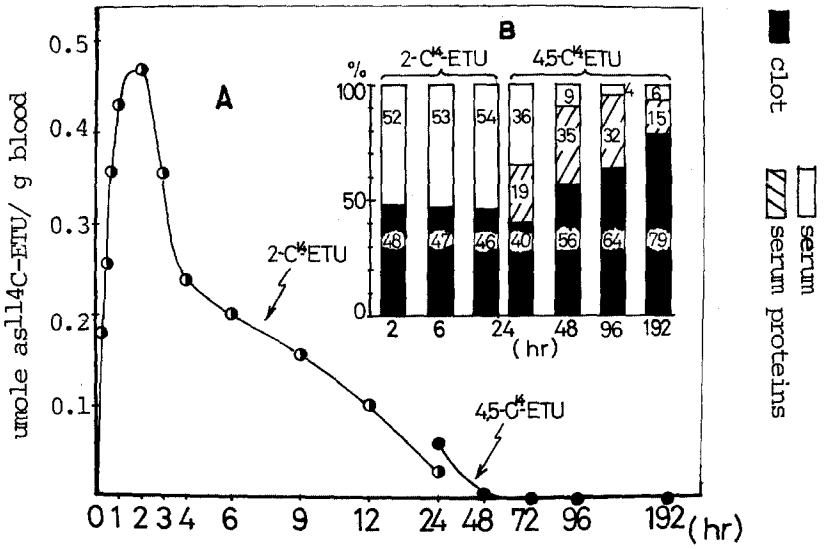

Fig.1 Radioactive substances in the blood. A--Radioactive concentration. B--Distribution of radioactivity in the blood.

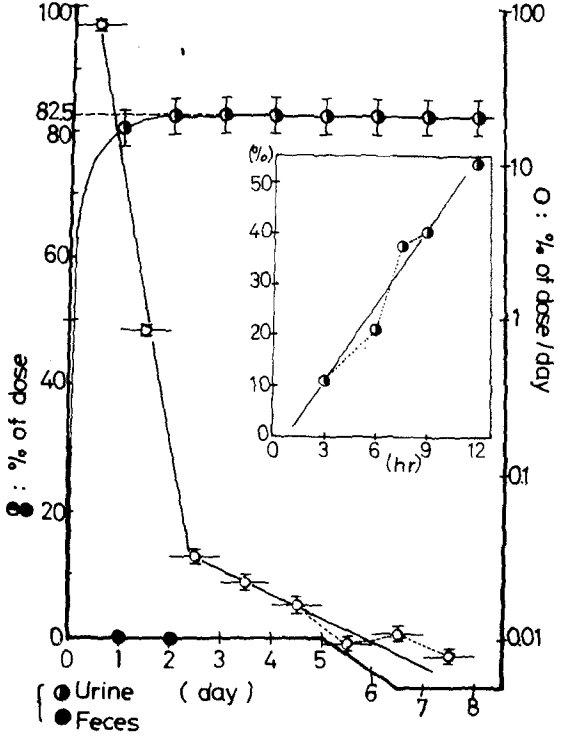

Fig. 2 Elimination into the urine and feces.

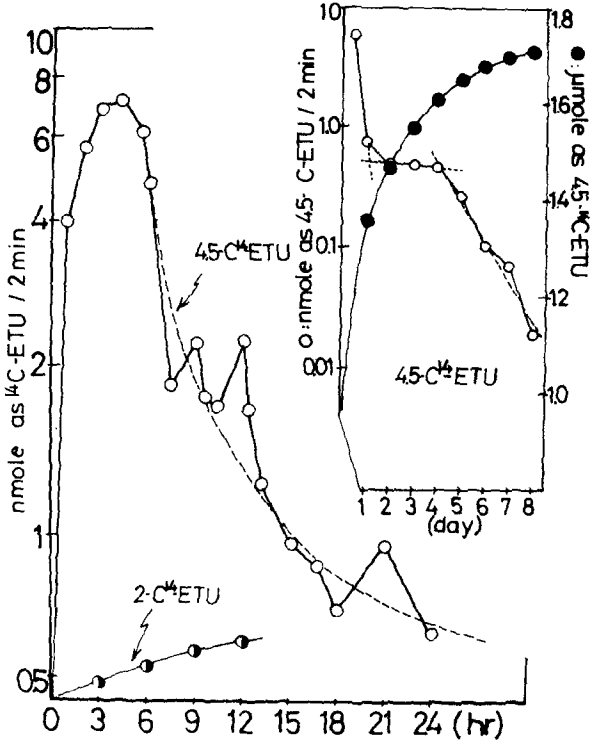

Fig. 3 Elimination to the expiration air.

DISTRIBUTION OF RADIOACTIVITY IN MATERNAL BLOOD. Radioactivity due to either type of the labeled ETU was found both in the serum and clot(fig.1). When 4,5-C^{14}-ETU was administered considerable portion of the activity in the serum was related to the serum crude protein fraction,and the percentage grew with the elapse of time. Further washing of the protein fraction with water,ethanol, acetone,and ether could not release any activity indicating the incorporation of 4 and/or 5 carbon atoms of ETU in proteins or other cell constituents. On the other hand in the case of 2-C^{14}-ETU any radioactivity could not be detected in the protein fraction.

RADIOACTIVE SUBSTANCES IN FETUS. Figure 4 shows the radioactive substances in the fetus,which were displayed by the amounts of equivalent C^{14}-ETU per fetus and their concentration. Within 2 hr the radioactivity(amount and concentration) in the fetus reached maximal and thereafter it was decreased rapidly. The concentrations of radioactive substances estimated as 2-C^{14}-ETU reached 0.220 ± 0.003 umole/ g in 2 hr and fell down to 0.012 ± 0.003 umole/ g by 24 hr. The fractionation analyses indicated that radioactive carbon of 2-C^{14}-ETU was not incorporated in the fetus protein fractions. About 71 to 73% of the radioactivity in intact fetuses were recovered in ethanol-water fraction(63-69%) and chloroform fraction(3.4-8%). Remaining 27 to 29% of the activity were lost during the fractionation processes. On the other hand,when 4,5-C^{14}-ETU was administered,about three fold of the radioactivities was found in the fetus of 24 hr after the dosage. In this case one third of the activity was due to that incorporated in the protein fraction. And the radioactivities found in the fetuses of 2 and 4 days after the dosage were substantially due to those incorporated in the protein fractions.

RADIOACTIVE SUBSTANCES IN OTHER TISSUES. Radioactivities due to 2-C^{14}-ETU were distributed comparatively homogeneously in almost all the tissues except the thyroid gland at approximately the same level as in the serum,and the radioactive concentrations were reduced to about less than 10% of their maximal levels (2 hr). However,in the thyroid gland the radioactive concnetration was much higher than the other tissues and increased constantly at least during the first 24 hr (tab.1)indicating the accumulative property of ETU itself or its metabolite(s) in the gland.

THIN LAYER CHROMATOGRAPHY OF FETUS EXTRACT. Figure 5 shows examples of thin layer radiochramatograms of fetus samples fed with 2-C^{14}-ETU. As early as in 2 hr considerable quantities of radioactive metabolites were transferred to the fetus, and in addition to ETU, 8 radioactive metabolites were detected.

Gaschromatographic analysis of the fetus extracts also indicated the transplacental passage of ETU(fig.6).

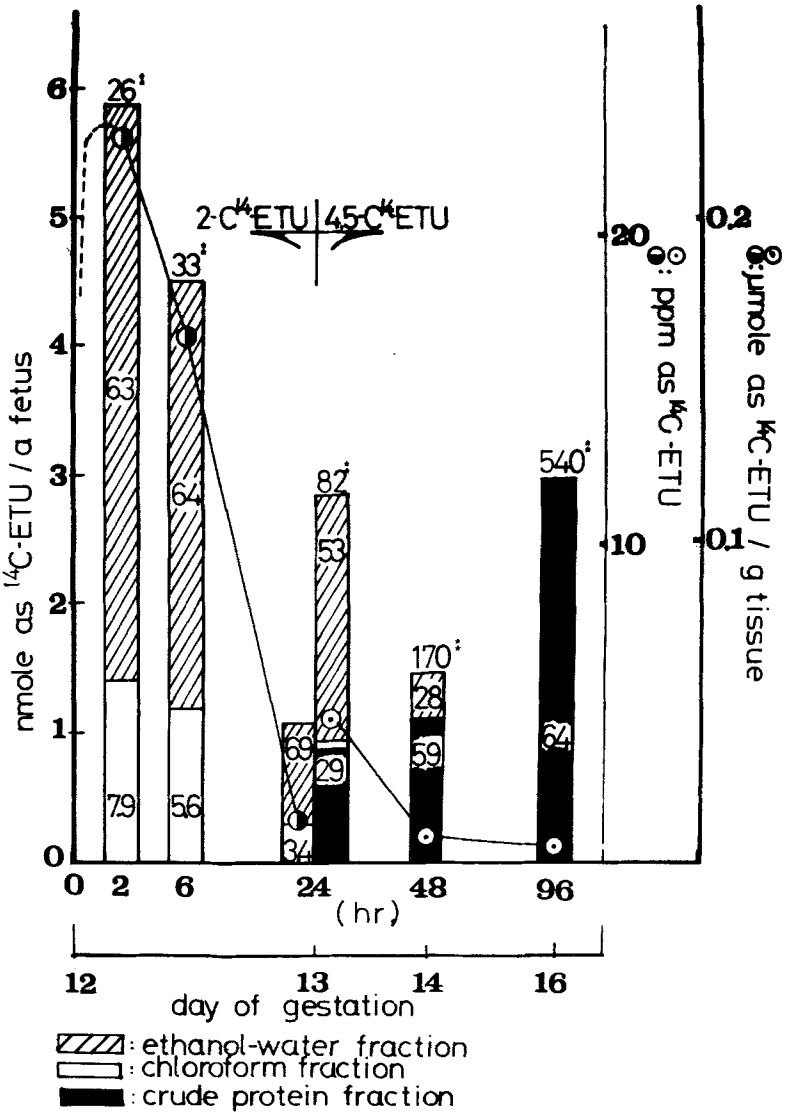

Fig. 4 Radioactive substances in the fetus.

* : mean fetus weight.

Each value in the bar is the distribution ratio (%) of radioactivity in the fraction to the total radioactivity in the fetus.

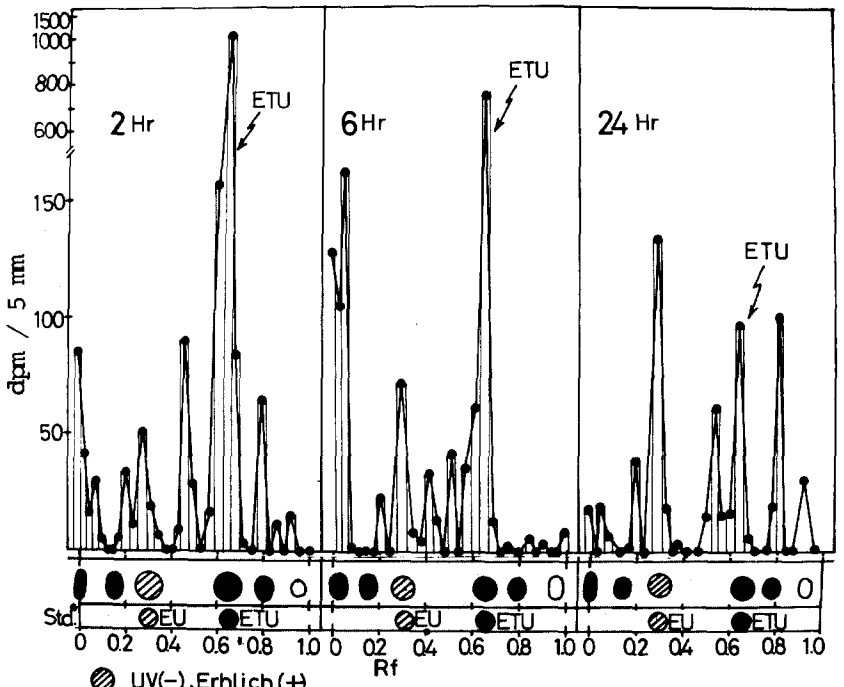

Fig.5 Thin layer radiochromatograms of fetus extracts.

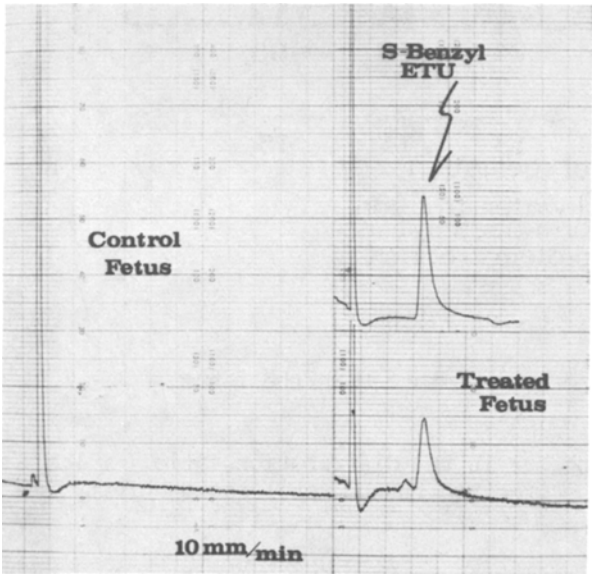

Fig.6

Gaschromatograms of fetus extracts.

Table 1.
Distribution of ^{14}C in rat tissues after the single oral dosage of 2-^{14}C-ETU (100 mg / kg, 935 umole / kg)

Tissue	^{14}C (umole as 2-^{14}C-ETU / g tissue)		
	2 hr*	6 hr*	24 hr*
Brain	0.245 ± 0.009	0.195 ± 0.027	0.011 ± 0.002
Lung	0.194 ± 0.019	0.136 ± 0.017	0.009 ± 0.002
Muscle	0.254 ± 0.012	0.207 ± 0.012	0.011 ± 0.004
Liver	0.204 ± 0.026	0.139 ± 0.043	0.021 ± 0.003
Spleen	0.183 ± 0.015	0.142 ± 0.019	0.010 ± 0.002
Kidney	0.243 ± 0.035	0.178 ± 0.019	0.014 ± 0.006
Thyroid gland	0.346 ± 0.100	0.483 ± 0.189	0.651 ± 0.034
Thymus gland	0.165 ± 0.021	0.153 ± 0.013	0.013 ± 0.002
Bone marrow	0.208 ± 0.022	0.155 ± 0.020	0.014 ± 0.001
Adrenal body	0.221 ± 0.054	0.116 ± 0.014	0.016 ± 0.002
Amniotic fluid	0.312 ± 0.086	0.166 ± 0.010	0.012 ± 0.003
Placenta	0.101 ± 0.017	0.054 ± 0.015	0.009 ± 0.002
Serum	0.352 ± 0.017	0.255 ± 0.017	0.022 ± 0.007
Fetus	0.220 ± 0.031	0.163 ± 0.028	0.014 ± 0.002

* Time after the dosage. Values are the means ± S.D. of 4 rats. Values concerning the fetus are the means ± S.D. of 16 fetuses of 4 litters.

DISCUSSION

Teratogenic dose of ETU was absorbed easily from rat gastrointestinal tract,translocated into the whole body tissues including the fetus at approximately homogeneously except the thyroid gland,and eliminated mostly into the urine very rapidly. These tendencies were also confirmed by whole body auto radiography (M.HIRANO and Y.KATO,unpublished),which showed high density of black deposits in the thyroid gland and homogeneous and lower density in the other tissues. This accumulative property of ETU in the thyroid gland had been also documented by NEWSOME(1974) using cold ETU in male rats and guinea pigs,and suggests that a single oral dosage of ETU induciable congenital malformations also causes the impairment of thyroid functions because of its known antithyroid property. GRAHAM and HANSEN(1972) reported that ETU repressed the uptake of ^{131}I by the thyroid gland at the dosage of 50 ppm for 30 days. Thyroid hormones are known to play important roles in the development of the central nervous system and thyroidectomy induces malformations in rats(LANGMAN and FAASEN,1955). From these findings a possible mode of teratogenic action of ETU may be explained by the impairment of thyroid function. However, the radioimmumoassay analyses of thyroxine(T_4) in the maternal serum did not show any significant difference in the T_4 levels between the treated rats(100mg/kg,single oral dosage on day 12 of gestation) and the controls ,whereas the apparence of malformed fetus was critical between the controls(0%) and the treated rats (gross malformation; 100%).

Table 2.

Thyroxine (T_4) levels in the maternal serum.

Day of gestation	Hour after the administration	Thyroxine level (ng / ml) control	treated
12	2	--------	37.1 ± 6.4
	6	--------	33.0 ± 19.9
13	24	41.1 ± 10.1	34.0 ± 8.4
14	48	33.5 ± 19.3	46.6 ± 15.2
16	96	41.2 ± 9.3	40.2 ± 7.0
20	192	50.1 ± 12.1	93.9 ± 25.5

Values are the means ± S.D. of four rats.
T_4 levels were estimated by using ResOmat T_4 kits.

In this study we demonstrated the existence of ETU and its metabolites in the fetus and the concentration of them reached maximal within 2 hr after the dosage in parallel to the maternal tissues except the thyroid gland and then it was decreased to a negligible level by 24 hr. The localization of the radioactivity in the fetus was not investigated in this study. In consideration of the accumulative property of thiourea in the fetus thyroid gland in rats (ULLBERG and HAMMARSTRÖM, 1969), antithyroid ETU may be also localized in the fetus thyroid gland.

As for the matabolic process of ETU, the results of the analyses on the expiration of radioactive carbon dioxide indicatted that ETU was metabolized to carbon dioxide via the fragmentation of imidazolidine ring and the the decarboxylation step of 4 and/or 5 carbon atoms of ETU was involved. However, the process involving the S-oxidation and decarboxylation of 2-carbon atom of ETU could not be well estimated because of the trace level of the expiration. In the thin layer chromatography, a metabolite which showed the same Rf value and reactivity with Ehrlich's reagent and UV absorvancy as ethyleneurea, a known photodegradation product of ETU (CRUICKSHANK and JARROW, 1973), was detected. But it was not identified in this work.

Further investigations on the metabolic fate of ETU are needed to clarify the mode of teratogenic action of ETU.

ACKNOWLEDGEMENTS

The authors thank to Dr. Masateru Uchiyama of Tokyo University of Agriculture and Technology for his courteous sdvices on the preparation of C^{14}-ETUs. We are also indebted to Dr. Shinko Goto, Director of Chemistry Division, and Dr. Yasuhiko Shirasu, Director of Toxicology Division, Institute of Environmental Toxicology, for their encouragement during the course of this work. This study was supported by the glant from The Ministry of Agriculture and Forestry.

SUMMARY

Metabolic fate of ETU was investigated in the rats administered orally 100 mg/ kg of C^{14}-ETU on the twelfth day of gestation.

ETU was absorbed readily from the gastrointestinal tract and passed away from the whole body tissues including the fetus rapidly. Only the exception was the thyroid gland and the radioactivity was accumulated in the gland. Most of the administered activity(80.2%,4,5-C^{14}-ETU) was eliminated into the urine in 24 hr and the tissues(including the fetus)levels of radioactivity from 2-C^{14}-ETU reached maximal within 2 hr and fell down to negligible levels by 24 hr.

Radiocarbon(s)of 4,5-C^{14}-ETU was expired as radioactive carbon dioxide and was incorporated into the serum and fetal cell constituents(crude protein fraction),but that of 2-C^{14}-ETU was neither expired or incorporated into the cell constituents.

From the fetus extract ETU and several radioactive metabolites were detected.

REFERENCES

ALLEN,J.F., et al., Organic Synthesis,vol.3,pp 394,New York, John Wiley & Sons,1967

CRUICKSHANK,P.A., and JARROW,H.C., J.Agr.Food.Chem.,21,333,(1973)

FAO / WHO : 1967 Evaluations of Some Pesticide Residues in Food, pp.179,Rome,1968

GRAHAM,S.L., and HANSEN,W.H., Bull.Environ.Contam.Toxicol.,7,19, (1972)

HYLIN,J.W., ibid., 10,227,(1973)

INNES,J.R.M., et al.,J.Natl.Canc.Inst., 42,1101,(1969)

KHERA,K.S., Teratology, 7,243,(1973)

LANGMAN,J., and FAASEN,F., Amm.J.Ophthalmol., 40,65,(1955)

NEWSOME,W.H., Bull.Environ.Contam.Toxicol.,11,174,(1974)

NEWSOME,W.H., and LAVER,G.W., ibid., 10,151,(1973)

SEILER,J.P., Mutation Res., 26,189,(1974)

TERAMOTO,S., et al., Congenital Anomalies(Japan,in preparation)

ULLAND,B.,et al., J.Natl.Canc.Inst., 49,583 (1972)