Ivie, G. W., Casida, J. E., *J. Agric. Food Chem.* **19**, 405 (1971a).
Ivie, G. W., Casida, J. E., *J. Agric. Food Chem.* **19**, 410 (1971b).
Ivie, G. W., Knox, J. R., Khalifa, S., Yamamoto, I., Casida, J. E., *Bull. Environ. Contam. Toxicol.* **7**, 376 (1972).
Khan, M. A. Q., Rosen, J. D., Sutherland, D. J., *Science* **164**, 318 (1969).
Khan, M. A. Q., Stanton, R. H., Reddy, G., in "Survival in Toxic Environments", Khan, M. A. Q., Bederka, J. P., Ed., Academic Press, New York, 1974.
Matsumura, F., Patil, D. C., Boush, G. M., *Science* **170**, 1208 (1970).
Nohynek, G. J., Mueller, W. F., Coulston, F., Korte, F., prepublication review of the paper entitled, "Metabolism, Excretion and Tissue Distribution of [^{14}C]Photodieldrin in Non-human Primates following Oral Administration and Intravenous Injection", submitted to *J. Agric. Food Chem.* (1978).
Parlar, H., Korte, F., *Chemosphere* **6**, 665 (1977).
Podowski, A. A., Banerjee, B. C., Feroz, M., Dudek, M. A., Willey, R. L., Khan, M. A. Q., *Arch. Environ. Contam. Toxicol.*, to be published (1978).
Reddy, G., Khan, M. A. Q., *J. Agric. Food Chem.* **22**, 910 (1974).
Reddy, G., Khan, M. A. Q., *J. Agric. Food Chem.* **23**, 861 (1975).
Reddy, G., Khan, M. A. Q., *J. Agric. Food Chem.* **25**, 25 (1977a).
Reddy, G., Khan, M. A. Q., *Gen. Pharmacol* **8**, 285 (1977b).
Reddy, G., Khan, M. A. Q., *J. Agric. Food Chem.* **26**, 292 (1978).
Rosen, J. D., Sutherland, D. J., Khan, M. A. Q., *J. Agric. Food Chem.* **17**, 404 (1969).
Rosen, J. D., Sutherland, D. J., Lipton, G. R., *Bull. Environ. Contam. Toxicol.* **1**, 127 (1966).
Turner, B. C., Glotfelty, D. E., Taylor, A. W., *J. Agric. Food Chem.* **25**, 548 (1977).

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Comparative in Vitro Metabolism of Tetrachlorvinphos by the Soluble Fraction (105000*g*) from Sheep, Pig, and Cow Liver Homogenates

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The organophosphorus insecticide tetrachlorvinphos was incubated with the soluble fraction (105000*g*) from homogenates of sheep, pig, and cow livers. Dealkylation to a water-soluble metabolite desmethyl tetrachlorvinphos by a reduced glutathione dependent enzyme system was the major route of degradation. The rate of dealkylation followed the order cow ≃ sheep > pig. The water-soluble metabolite was further metabolized very effectively by the enzyme system in the soluble fraction. The metabolites 2,4,5-trichlorophenacyl chloride, 2,4,5-trichloroacetophenone, 1-(2,4,5-trichlorophenyl)ethanol, and 2-chloro-1-(2,4,5-trichlorophenyl)ethanol were positively identified. The rate of production of these metabolites did not vary greatly between species.

The metabolism of tetrachlorvinphos has been investigated in the rat and dog (Akintonwa and Hutson, 1967) and in the dairy cow (Gutenmann et al., 1971). In vitro studies with the supernatant (1000000*g*) from mammalian livers (mouse, rat, rabbit, and pig) have been reported (Hutson et al., 1972). More recently, Akhtar and Foster (1977) reported in vitro studies with the soluble fraction (105000*g*) from chicken liver homogenates. This study examines the soluble fractions (105000*g*) from the livers of sheep, pig, and cow for species differences in the mode and rate of degradation of the insecticide and reports the relative amounts of metabolites produced.

EXPERIMENTAL SECTION

Chemicals. Pesticide and reagent grade solvents were used. ^{14}C-labeled tetrachlorvinphos (I), desmethyl tetrachlorvinphos (II), 2,4,5-trichlorophenacyl chloride (III), 2,4,5-trichloroacetophenone (IV), 1-(2,4,5-trichlorophenyl)ethanol (V), and 2-chloro-1-(2,4,5-trichlorophenyl)ethanol (VI) were prepared using a previously published procedure (Akhtar and Foster, 1977). *S*-(2,4,5-Trichlorophenacyl)glutathione was synthesized by the technique reported by Akhtar (1978).

Enzyme Preparation. Portions of livers were removed immediately after slaughter from pregnant Yorkshire sows approximately 2 years old, pregnant ewes from a new synthetic crossbred line approximately 3 years old, and cows approximately 4 years old, and were washed in distilled water and placed on crushed ice. The soluble fraction (105000*g*) was prepared in ice-cold 0.134 M phosphate buffer, pH 7.4 (8 g/40 mL), in a glass–Teflon homogenizer as described by Akhtar and Foster (1977). The precipitate (microsomes) after centrifugation at 40000 rpm (10500*g*) was washed by resuspending in buffer and centrifuging at 40000 rpm for a further 30 min. The resultant precipitate was the microsomal preparation and was resuspended in one-fourth of the original volume of phosphate buffer.

In Vitro Incubations. Incubations with the soluble fraction were carried out in a water-bath in glass-stoppered Erlenmeyer flasks (50 mL) at 37.5 °C under nitrogen. The incubation mixtures consisted of enzyme preparation (4.5 mL), reduced glutathione (0.5 mL), and [*vinyl*-^{14}C]-tetrachlorvinphos (122 μg). Reduced glutathione (GSH) was prepared just prior to addition by mixing 0.0027 g (8.7 × 10^{-6} mol) of glutathione with 0.5 N NaOH (0.05 mL) and neutralizing with 0.134 M phosphate buffer, pH 7.4 (0.45 mL). The reactions were carried out in duplicate and were terminated by addition of acetone.

Reactions with microsomal preparations were carried out in glass-stoppered Erlenmeyer flasks (25 mL) under O_2/CO_2 (95:5) at 37.5 °C. The volume of the reaction mixture was 3 mL and contained enzyme preparation (2.4 mL),

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Table I. Percentage of Original Radioactivity Recovered after Incubation with Soluble Fraction(105000g) from Sheep, Pig, and Cow Liver[a]

	radioactivity, %[b]					
	benzene			aqueous		
time, min	sheep	pig	cow	sheep	pig	cow
10	16.3	41.0	21.3	70.8	57.4	72.2
20	10.6	20.6	12.7	76.8	67.3	76.0
30	16.7	12.6	20.3	64.3	52.2	62.1
60	26.7	32.2	37.3	41.8	47.4	49.4
120	34.1	54.2	44.0	33.1	23.1	32.4
180	45.6	64.9	63.0	32.8	18.4	22.4
240	48.1	75.5	72.1	22.3	10.9	18.1

[a] Incubation mixture contained 4.5 mL of enzyme preparation, 122 μg of [^{14}C]tetrachlorvinphos, and 0.5 mL of freshly prepared GSH solution. The incubations were carried out under N_2 at 37.5 °C. [b] Percentage radioactivity is the average of duplicate incubations.

^{14}C-labeled tetrachlorvinphos (122 μg), and reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system (0.6 mL). The NADPH generating system consisted of 0.1 mL each of KCl (12.5×10^{-3} M), NADP (10^{-4} M), nicotinamide (5×10^{-3} M), glucose 6-phosphate (G-6-P) (5×10^{-3} M), G-6-P dehydrogenase (1.5 units), and ethylenediaminetetraacetic acid (EDTA) (10^{-3} M) (Ray, 1967). The reactions were carried out in duplicate and were terminated by addition of acetone.

Extraction of Metabolites. Incubation mixtures were filtered, and the residue was washed with acetone (120–130 mL), concentrated (5–7 mL), and extracted with benzene (Akhtar and Foster, 1977). The volumes of the aqueous and the benzene phases were adjusted to 5 mL. The radioactivity in each fraction was measured in duplicate aliquots (1 mL).

Measurement of Radioactivity. The radioactivity of the extracts was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3320, using an external standard and correcting the data for quenching. Benzene extracts were assayed in a scintillation solution containing 2,5-diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in toluene (5 g, 50 mg, 1000 mL), whereas the radioactivity in aqueous fractions was measured in PCS (Amersham/Searle), a xylene-surfactant liquid scintillation cocktail.

Gas Chromatography (GC). Benzene extracts were analyzed on a Packard-Becker 420 gas chromatograph equipped with ^{63}Ni electron-capture detector. The column was a 1.83 m × 4 mm (i.d.) glass tube packed with 3% SE-30 on 80–100 mesh Chromosorb WHP. The operating temperatures for injector, column, and detector were 135, 130, and 295 °C, respectively; 5% methane/argon (column flow 30 mL/min, purge 15 mL/min) was the carrier gas. Under the GC conditions described, the relative retention times for I, III, IV, V, and VI were 1.80, 0.67, 0.46, 0.56, and 1.0, respectively.

Thin-Layer Chromatography (TLC). Silica gel OF plates (New England Nuclear) were developed in ethyl acetate/hexane (3:7, v/v) and the compounds were located by viewing under UV light (254 nm). The R_f values for I, III, IV, V, and VI were 0.31, 0.82 0.78, 0.62, and 0.59, respectively. Silica gel G plates were used for the analyses of water-soluble metabolites. Compound II had an R_f value of 0.41 in methanol/isopropyl alcohol/acetone (1:1:8, v/v/v), whereas *S*-(2,4,5-trichlorophenacyl)glutathione had an R_f value of 0.64 in 1-butanol/acetic acid/water (11:5:4, v/v/v).

Gas Chromatography–Mass Spectrometry (GC–MS). The GC–MS analysis was accomplished in a Finnigan Model 3100 mass spectrometer connected to a Finnigan Model 9500 gas chromatograph by means of a jet separator. The mass spectrometer was interfaced with a Model 6100 computer-controlled data acquisition system. A 1.52 m × 4 mm (i.d.) glass column packed with 3% SE-30 on 80–100 mesh Chromosorb WHP was used for gas chromatographic separation. The column was run at 175 °C and the helium flow was 35 mL/min. The mass spectra were recorded at 70 eV.

RESULTS AND DISCUSSION

The radioactivity distribution data from incubation of ^{14}C-labeled tetrachlorvinphos with the soluble fraction (105000g) from pig, sheep, and cow liver homogenates are listed in Table I. In each case the insecticide was initially degraded to a water-soluble product as can be seen from the loss of radioactivity in the benzene layer and a concomitant, but not equal, increase in the aqueous phase. It is also evident that both cow and sheep liver preparations metabolized the insecticide at approximately the same rate, whereas metabolism with pig liver preparation was considerably slower. In 10 min 70–72% of the insecticide was converted to water-soluble product(s) by sheep and cow preparation while only 57% was converted by that from pig.

Radioactivity in the aqueous phase reached a maximum at 20 min, then gradually decreased with a concomitant increase in radioactvity in benzene fraction. This suggests that the water-soluble metabolite(s) was being degraded to benzene-extractable products and probably involved multiple steps. This is consistent with the findings reported for chicken liver soluble fraction (Akhtar and Foster, 1977).

Initially, the radioactivity in the aqueous phase was associated with a single metabolite, desmethyl tetrachlorvinphos. However, on prolonged incubation, a second radioactive region was detected on the plate very near the origin. This radioactive area was extracted with acetone/water (1:1) and exhibited a ninhydrin positive spot on TLC at R_f 0.65 in 1-butanol/acetic acid/water (11:5:4, v/v/v). When this eluted material was incubated with enzyme preparation in the presence of GSH or 2-mercaptoethanol, 1-(2,4,5-trichlorophenyl)ethanol was produced. This compound was also formed when *S*-(2,4,5-trichlorophenacyl)glutathione was incubated with enzyme preparation with sulfhydryl(s) (Akhtar, 1978). Therefore, the identity of this radioactive area has been established as *S*-(2,4,5-trichlorophenacyl)glutathione. In the present study, however, no attempt was made to quantitate the compound.

The benzene phase of incubations for up to 30 min contained unmetabolized I and metabolites III, IV, V, and VI. However, III and IV were undetected in the 60-min benzene phase. The benzene extracts after 60 min con-

Table II. Concentration of 1-(2,4,5-Trichlorophenyl)ethanol in Incubation Mixtures at Various Time Intervals

time, min	concentration, μg[a] sheep	pig	cow
10[b]	3.7	2.4	1.3
20[b]	7.0	10.5	2.2
30[b]	13.3	18.4	2.6
60[c]	15.4	28.7	17.7
120[c]	31.4	32.5	29.5
180[c]	37.1	45.3	39.9
240[c]	39.5	54.5	52.3

[a] Concentrations are the average of duplicate incubations. [b] Incubation mixtures also contained metabolites III, IV, and VI. [c] Incubation mixtures also contained metabolite VI.

Table III. Role of Reduced Glutathione (GSH) in the Metabolism of Tetrachlorvinphos (I)

conditions	% I metabolized[a] sheep	cow	pig
I + enzyme[b]	80	84	80
I + enzyme[b] + GSH (8.7×10^{-6} mol)	91	93	93
I + enzyme[c]	13	19	18
I + enzyme[c] + GSH (3.5×10^{-5} mol)	92	95	91
I + enzyme[d]	12	11	13
I + enzyme[d] + GSH (3.5×10^{-5} mol)	90	90	92

[a] Percentage metabolized is the average of duplicate incubations. The incubation mixture contained 4.5 mL of enzyme preparation, 122 μg of [^{14}C]tetrachlorvinphos and either 0.5 mL of 0.134 M phosphate buffer, pH 7.4, 0.5 mL of GSH solution. [b] Undialized. [c] Dialyzed against 0.134 M phosphate buffer, pH 7.4 at 4 °C for 14 h. [d] Ammonium sulfate (80% saturation) fractionated preparation.

sisted mainly of metabolite V with small amounts of VI (Table II). Compounds I, III, IV, V, and VI were positively identified by comparison of TLC R_f data, GC retention times, and GC–MS data with similar data from authentic standards. The GC–MS data of I, III, IV, V, and VI exhibited molecular ion peaks at m/e 364, 256, 222, 224, 258, respectively.

If I was incubated with dialyzed soluble fractions, only partial (11–19%) metabolism occurred (Table III). This degradation may be due to the action of tissue-bond GSH residues or to the direct hydrolysis of the P–O–vinyl bond. The addition of freshly prepared GSH (3.5×10^{-5} mol) to the incubation media restored the activity of the enzyme. Thus, dealkylation to desmethyl tetrachlorvinphos follows the P–O–CH_3 cleavage, which involves the GSH-dependent enzyme system, *S*-transferase. This is in agreement with the observations of Hutson et al. (1972) who reported that mammalian (mouse, rat, rabbit, and pig) liver contains a GSH-dependent soluble enzyme capable of degrading the CH_3–O–P bond in dimethyl phosphoric acid triester (tetrachlorvinphos etc.) or dimethyl phosphorothionic acid triester.

Incubations of I with enzyme and GSH (8.7×10^{-6} mol) for 60 min produced metabolites V and VI; whereas reaction of I with dialyzed enzyme and GSH (3.5×10^{-5} mol) also resulted in a small amount of IV in addition to V and VI. In both cases V was the major metabolite, accounting for 85–90% of the product. On the other hand, incubation of I with $(NH_4)_2SO_4$ fractionated enzyme preparation and GSH (8.7×10^{-6} mol) caused only partial metabolism of I and produced all the four organoextractable metabolites

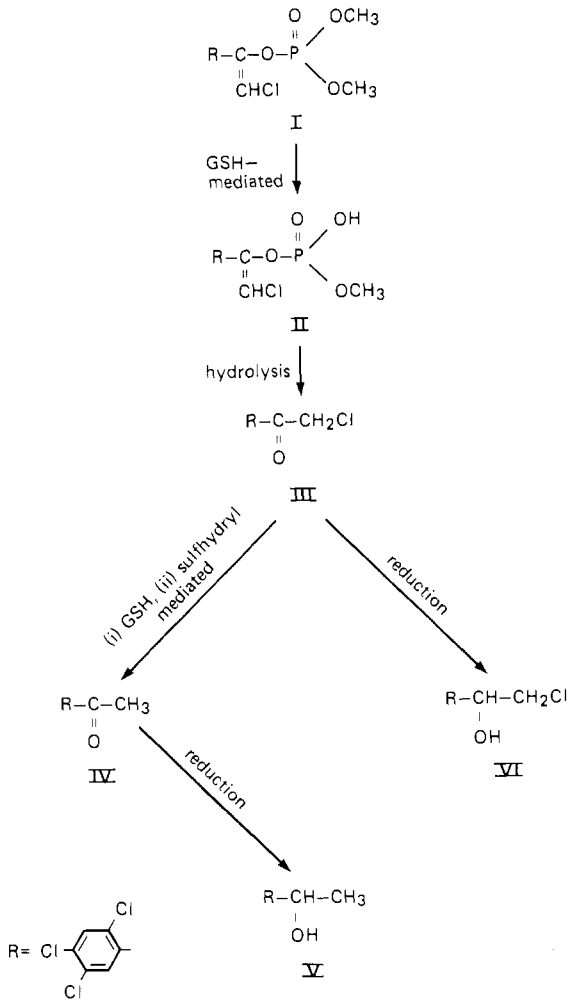

Figure 1. Metabolic pathways of tetrachlorvinphos in pig, sheep, and cow liver soluble preparation (105000*g*).

III, IV, V, and VI (approximately 3:2:4:1, by weight). Addition of excess GSH (3.5×10^{-5} mol) to the incubating media containing I and $(NH_4)_2SO_4$ fractionated enzyme efficiently metabolized I and afforded a mixture of IV and V in approximately 4 to 6 ratio (by weight) along with a small, but detectable amount of VI. These data indicate that both dialysis and $(NH_4)_2SO_4$ fractionation techniques either removed the reducing agent(s) or destroyed the enzyme reductase in addition to removal of GSH. However, $(NH_4)_2SO_4$ fractionation considerably lowered the reducing properties of the enzyme preparations. In the present study, however, no attempt was made to establish the role of NADP and NADPH in the formation of V and VI. From the above data it is also apparent that addition of excess GSH to the system containing $(NH_4)_2SO_4$ fractionated enzyme preparation not only restored the dealkylation step but also assisted in other metabolic pathways. This is evident by the complete absence of III in incubation mixture with excess GSH.

The early appearance of III and IV, and their subsequent disappearance from the incubation mixture, is an indication that further degradation takes place. This is identical and consistent with previous findings for chicken liver preparations (Akhtar and Foster, 1977). It has been shown previously (Akhtar, 1978) that III is converted to IV via a glutathione conjugate intermediate, *S*-(2,4,5-trichlorophenacyl)glutathione, which involves a sulfhydryl-dependent enzyme system(s). Metabolite IV is reduced enzymatically to V. The detection and identification of *S*-(2,4,5-trichlorophenacyl)glutathione in the

incubation media is an added proof of previous reports.

Contrary to a previous report for chicken liver preparations (Akhtar and Foster, 1977), addition of GSH (8.7×10^{-6} mol) to freshly prepared soluble fraction from sheep, cow, and pig livers produced only a small increase (approximately 10%) in the amount of I which was metabolized (Table III). This implies that the concentration of either GSH or *S*-transferase, or both, is higher in sheep, cow, and pig preparations than in that from chicken liver. This observation is in agreement with the report (Johnson, 1966) that dealkylation of iodomethane, under comparable conditions, by liver preparations from ox and pig was four to five times greater than by chicken liver.

Donninger et al. (1972) observed oxidative metabolism of I to desmethyl tetrachlorvinphos and formaldehyde by the microsomal preparation from dog liver and reported that 19 and 25% of I was metabolized in 30 min and 1 h, respectively. In the present study, when I was incubated with microsomal preparations from cow and pig liver, only 20–30 was converted in 4 h to a water-soluble metabolite that was identified as desmethyl tetrachlorvinphos. It appears that microsomal enzyme(s) has a less significant role in the metabolism of I. Therefore, this aspect was pursued no further.

The data presented show that all three species possess an effective GSH-dependent enzyme system(s), *S*-transferase(s), capable of degrading tetrachlorvinphos into a water-soluble metabolite. The soluble fraction (105000*g*) also contains enzyme systems such as hydrolase, dechlorinase, (probably sulfhydryl-dependent dechlorinase; Akhtar, 1978), and reductase. The data also indicate that in pig, cow, and sheep liver, it is the enzymes in the soluble fraction which are predominant in the metabolism of the insecticide, since microsomal enzyme metabolism proceeded much more slowly. Based on the details above, a metabolic pathway of tetrachlorvinphos by soluble fraction (105000*g*) from sheep, pig, and cow is shown in Figure 1. In vivo studies with the lactating cow are presently under investigation.

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LITERATURE CITED

Akhtar, M. H., Foster, T. S., *J. Agric. Food Chem.* **25**, 1017 (1977).
Akhtar, M. H., *J. Environ. Sci. Health*, in press (1978).
Akintonwa, D. A. A., Hutson, D. H., *J. Agric. Food Chem.* **15**, 682 (1967).
Donninger, C., Hutson, D. H., Pickering, B. A., *Biochem. J.* **126**, 701 (1972).
Gutenmann, W. H., St. John, L. E., Jr., Lisk, D. J., *J. Agric. Food Chem.* **19**, 1259 (1971).
Hutson, D. H., Pickering, B. A., Donninger, C., *Biochem. J.* **127**, 285 (1972).
Johnson, M. K., *Biochem. J.* **98**, 44 (1966).
Ray, J. W., *Biochem. Pharmacol.* **16**, 99 (1967).

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A Metabolite of Polybrominated Biphenyls: Its Identification and Decomposition to a Brominated Dibenzofuran in the Gas Chromatograph–Mass Spectrometer

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The feces of dogs fed Firemaster BP-6, a mixture of polybrominated biphenyls, contained a metabolite identified as 6-hydroxy-2,4,5,2′,4′,5′-hexabromobiphenyl (hydroxy-HBB). Mass spectrometry (MS), thin-layer chromatography (TLC), and gas chromatography (GC) were used to compare the metabolite and synthetic hydroxy-HBB. The substitution pattern of the synthetic hydroxy-HBB was determined by comparing its nuclear magnetic resonance spectrum with that of 2,4,5,2′,4′,5′-HBB. MS analysis showed that, during GC on OV-101 columns at 230 to 260 °C, hydroxy-HBB unexpectedly decomposed into two pentabromodibenzofurans (PDBF). The hydroxy compound could be differentiated from the PDBF artifacts by TLC, infrared spectroscopy, formation of an acetate derivative, and MS analysis, using direct probe insertion into the source. Similar tests should be used to corroborate GC–MS data that indicate the presence of brominated dibenzofurans as contaminants or metabolites.

Polybrominated biphenyls (PBBs) are chemically similar to polychlorinated biphenyls (PCBs), a class of widespread environmental pollutants (Hutzinger et al., 1974). PBB has been found in milk, eggs, meat, and human blood in Michigan due to inadvertent feed contamination with Firemaster BP-6, a flame retardant (Hoeting, 1976). Firemaster BP-6 is a PBB mixture with 2,4,5,2′,4′,5′-hexabromobiphenyl (HBB) as its major component (Sundström et al., 1976a; Jacobs et al., 1976). (See Figure 1A for numbering of substituent positions in the biphenyl nucleus.)

It is often desirable to study the animal metabolism of industrial chemicals found as food contaminants because the metabolites may be more toxic than the parent materials. While examining fecal extracts for possible toxic metabolites of PBB by gas chromatography–mass spectrometry (GC–MS), we observed a substance having a mass spectrum of a brominated dibenzofuran (DBF). (See Figure 1B for dibenzofuran structure.) Certain chlorinated DBFs are known and are very toxic compounds (Kimbrough, 1972; McKinney et al., 1976). They have been found as contaminants of PCB (Bowes et al., 1975) and there is evidence that they were excreted in the urine of

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