Bovine Metabolism of Organophosphorus Insecticides. Metabolism and Residues Associated with Dermal Application of Co-ral to Rats, a Goat, and a Cow

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Co-ral was applied dermally to rats, a cow, and a goat at 30 to 45 mg. per kg. The animals were sacrificed at predetermined intervals and the tissues were tested, chromatographically, for residues of the insecticide. Other factors investigated were the in vitro and in vivo opening of the pyrone ring in Co-ral and its oxygen analog, the ease of extraction of Co-ral and the oxygen analog from proteinaceous solutions, levels of Co-ral and metabolites appearing in blood, and the effect on the blood cholinesterase activity, milk residues, and the nature of the products excreted in the urine and feces.

Co-ral or Bayer 21/199 [*O*-(3-chloro-4-methylumbelliferone) *O,O*-diethyl phosphorothioate], has shown considerable promise for systemic cattle grub control following dermal administration (*17, 18*). Brundrett, McGregor, and Bushland (*3*) demonstrated that 0.75% sprays of Co-ral either as an emulsion or suspension almost completely prevented *Hypoderma lineatum* (DeVill). grubs from encysting in the backs of infested cattle. Co-ral is of relatively low toxicity to mammals (*1, 6*) and cows can be safely sprayed at this dose with no more than mild transitory reduction in blood cholinesterase activity (*15*). Radiotracer studies have shown that only a small portion of the Co-ral applied to cattle is absorbed and that tissue residues are very low 2 weeks after treatment (*16*). Lindquist *et al.* (*11*) have recently shown that rats rapidly metabolize and excrete a large proportion of a 20-mg.-per-kg. oral dose of Co-ral.

Radioactive Co-ral was administered to rats, a goat, and a cow in order to study the metabolic pathway and tissue residues associated with the use of this animal systemic insecticide.

Methods and Results

Chemical Studies

Synthesis of Radioactive Co-ral and Its Oxygen Analog. Co-ral was prepared from phosphorus-32 trichloride by the general method of Hein and McFarland (*9*) except that 3-chloro-4-methylumbelliferone was used instead of *p*-nitrophenol. The product was dissolved in chloroform, washed three times with 10% aqueous sodium carbonate and purified by partition chromatography on a Celite–iso-octane–methanol column (*2*). The radioactive material was identical in infrared spectrum to known *O*-(3-chloro-4-methylumbelliferone) *O,O*-diethyl phosphorothioate. Another sample of phosphorus-32–labeled Co-ral was 99% pure based on alumina column chromatography and infrared determination. The oxygen analog of Co-ral was prepared by oxidizing radioactive Co-ral with anhydrous peracetic acid in chloroform and purifying the product on an alumina column. Infrared spectrum analysis was identical with that of known oxygen analog.

Adsorption and Ion Exchange Chromatography. Hydrolysis products of Co-ral were separated by ion exchange chromatography using the system described for hydrolysis products of *O,O*-dimethyl *O*-(2,4,5-trichlorophenyl) phosphorothioate (*12*). Adsorption chromatography was used in the purification and identification of Co-ral and its oxygen analog. To aluminum oxide (Woelm, acid, activity grade I for chromatographic analysis) was added 2% by weight of water, and the hydrated aluminum oxide was immediately suspended in Skellysolve B and packed into a column. Neither Co-ral nor its oxygen analog were eluted from this column with Skellysolve B, Co-ral was eluted with benzene and the oxygen analog with chloroform. The columns were washed with methanol to ascertain that all phosphorus-containing compounds had been eluted.

Alkaline Hydrolysis of Co-ral and Derivatives. The nature of the products formed from reaction of Co-ral with ethanolic potassium hydroxide were studied. An absolute ethyl alcohol solution of potassium hydroxide was added to an equal volume of acetone containing Co-ral or its oxygen analog. The mixture with 100 μmol. of organophosphate per ml. was held at 28° C. for 48 hours, and the solvent was evaporated. The reaction with two molar equivalents of potassium hydroxide under the described conditions completely converted Co-ral to water-soluble derivatives, probably due to the opening of the pyrone ring to form the di-potassium salt of the corresponding coumarinic acid. This salt was not extractable into chloroform from aqueous solution but on acidification of the aqueous solution a complete reversion to Co-ral was obtained (*19*) based on the infrared spectrum and toxicity to flies (*10*) of the recovered product. Similar results on the opening and closing of the pyrone ring were obtained with the oxygen analog of Co-ral and with 3-chloro-4-methylumbelliferone. No evidence was obtained for the formation of a coumaric acid or benzofuran derivative (*8*) of Co-ral under these conditions.

The reaction mixture from Co-ral and three molar equivalents of potassium hydroxide were dissolved in water, acidified to close the ring, and extracted with chloroform; and the products remaining in the aqueous phase were separated by ion exchange chromatography. The only product found was tentatively identified as the de-ethylated derivative, *O*-(3-chloro-4-methylumbelliferone) *O*-ethyl phosphorothionic acid as, on the ion exchange column, it was separable from other possible hydrolysis products and eluted by the solvent system previously shown to elute the dealkylated product of Trolene (*13*) and other organophosphates (*14*). The oxygen analog with three molar equivalents of potassium hydroxide yielded 25% of the de-ethylated derivative and 75% diethyl phosphoric acid. Six molar equivalents of potassium hydroxide yielded only diethyl phosphorothioic acid and diethyl phosphoric acid with Co-ral and its oxygen analog, respectively.

The extent to which fission of the pyrone ring in Co-ral and its oxygen analog occurred at different hydrogen ion concentrations was determined using a series of 0.1 molar borate buffer solutions. Preliminary to this study the solubility of radioactive Co-ral, determined in water, was 1.5 p.p.m. and that of the radioactive oxygen analog was 25 p.p.m. To 10-ml. buffer solutions of varying pH were added low levels of Co-ral and its oxygen analog

in 0.2 ml. acetone. The solutions of organophosphates were incubated at 28° C. for 4 hours.

A chloroform extraction was then used to determine the proportion of water-soluble reaction products (salts of the corresponding coumarinic acids formed through fission of the pyrone ring). The percentages of apparent open ring at various pH levels were as follows: pH 7.5, 8%; pH 8.0, 13%; pH 8.5, 38%; pH 9.0, 87%; pH 9.5, 98%; and pH 10 to 12, 100%. The oxygen analog showed identical results. Longer periods of incubation had little effect in increasing the open pyrone ring.

The water-soluble products were acidified with concentrated hydrochloric acid and repartitioned with chloroform to determine the time necessary to close the pyrone ring. At pH 1.0 and 28° C., the ring closed rather slowly, required approximately 30 minutes for completion. On the other hand, the opening of the pyrone ring in alkali (pH 10 to 12) was almost instantaneous. This conclusion is based on visual observation of solubilization as the salts of the coumarinic acids were formed, and on the very short time of exposure necessary for Co-ral at pH 10 to 12 before it could no longer be extracted into chloroform. The oxygen analog showed identical results to those described for Co-ral. At pH 12, 5% hydrolysis occurred in 4 hours at 28° C. with Co-ral and 50% with the oxygen analog based on inability to recover these proportionate amounts of the organophosphates by chloroform extraction of aqueous media after closing the pyrone ring through acidification. No hydrolysis occurred below pH 11 within the 4-hour period.

Total phosphorus (*4*) and radioactive measurements (*13*) were made by previously described procedures. Infrared spectra were obtained with a Baird spectrophotometer with sodium chloride optics and 10% solutions of the chemicals in chloroform.

Ease of Extraction of Co-ral and Oxygen Analog from Protein Solutions. One hundred micrograms of radioactive Co-ral or its oxygen analog were coated uniformly on the sides of 15-ml. centrifuge tubes, 1.0-ml. portions of various protein solutions were added, and the tubes were agitated on a mechanical shaker at 37° C. for periods up to 24 hours. A small wooden stick added to each tube created a swirling action within the solutions for better mixing. This 1.0-ml. amount of protein solution came in contact with the total deposit of radioactive material. The change in ease of extraction of the radioactivity into an equal volume of chloroform was determined (Figure 1). When the chloroform was added immediately after placing the protein solutions into the tubes, the radioactivity was all recovered in the chloroform layer.

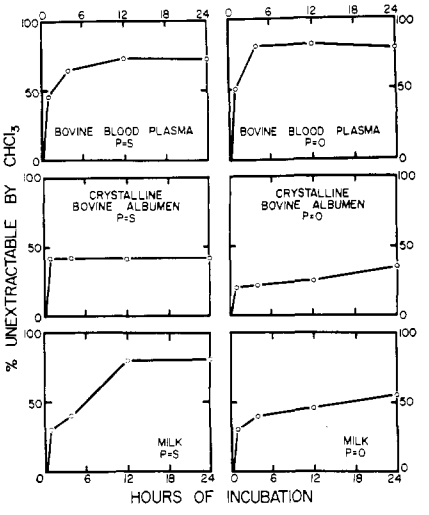


Figure 1. Ease of extraction with chloroform of Co-ral and its oxygen analog from protein solutions after various incubation times

Within a few hours a large proportion of the radioactivity was no longer extractable into chloroform. Generally, more Co-ral remained in the aqueous phase than oxygen analog. Identical results from both the phosphate and phosphorothioate were obtained from fresh bovine plasma, and bovine plasma pre-incubated at 28° C. for 4 days in the presence or in the absence of 0.1*M* fluoride ion. With Co-ral, the loss in ease of extraction into chloroform occurred at a similar rate with bovine serum (Nutritional Biochemicals Corp.) as with fresh bovine plasma. Crystalline bovine and egg albumen as 7% solutions in physiological saline produced similar effects. Even with fresh whole milk, the ease of extraction into chloroform diminished within a few hours.

With all the crystalline proteins and biological fluids studied, 20 to 50% of the radioactivity remaining in the aqueous layer could be recovered with the first chloroform extraction by incubation of the water-protein phase at pH 1.0 for 24 hours and then by re-extracting with chloroform. An additional 2 to 20% could be released from the aqueous phase with 48 hours more of incubation at pH 1.0 and another chloroform extraction.

Some Co-ral hydrolysis occurred in fresh bovine plasma as diethyl phosphorothioic acid was recovered by ion exchange chromatography from the water-soluble fraction following incubation of Co-ral at 250 p.p.m. in whole bovine plasma for 4 hours at 37° C. and extraction with chloroform. However, enzymatic hydrolysis of Co-ral or its oxygen analog could not explain all of the results, as crystalline bovine and egg albumin gave similar responses to fresh bovine plasma. Furthermore, the loss in ease of extraction with chloroform was the same with fresh bovine plasma as with bovine plasma held 4 days at 28° C. in the presence of high fluoride ion concentration. It further seems improbable that an opening of the pyrone ring was involved as the pH of the protein solutions was below that necessary for such pronounced results. Enzymatic action to open the pyrone ring seems unlikely because the effects noted also occurred with nonenzymatic proteins and in the presence of high fluoride ion concentration.

The effect is probably due to a change in the availability of the Co-ral or oxygen analog in the protein solutions for extraction. The radioactivity did not remain on the glass during the extractions as it could be totally recovered in the liquid layers assayed. In zero time analyses, the chloroform was added immediately after the protein solution so that the radioactive material would not yet have been dispersed in the protein solutions. Little change in ease of extraction occurred after the first 30 minutes. Emulsification of the radioactive materials by the protein solutions may have contributed to the results, as when Co-ral or its oxygen analog was emulsified in water using Triton X-100, it was difficult to recover them by extraction with chloroform. Other possible explanations might be binding on or occlusion in the proteinaceous material.

Co-ral Distribution and Metabolism in Rats

Administration of Co-ral and Derivatives to Rats. Radioactive Co-ral was administered to 250-gram male white rats subcutaneously, dermally, and orally at 40, 45, and 50 mg. per kg., respectively. Corn oil solutions were used for oral and subcutaneous treatments. The rats were sheared 3 days prior to dermal application. One milliliter of Co-ral emulsion containing 40% xylene and 3.5% Triton X-100 was applied to each rat using a syringe and blunt-tipped hypodermic needle. The application site extended about an inch on either side of the spinal column from the neck to the tail. Immediately following application, a 2-inch-square celluloid collar was stapled around the neck of the rat and left on for 72 hours to prevent the rat from licking the treated area.

Metabolism cages (*7*) were used for extraction studies with four rats for each method of treatment. One drop of toluene was added to the urine samples and they were held at −10° C. until analyzed by ion exchange chromatography.

Thirty 250-gram male rats were treated dermally with 45 mg. per kg. of radioactive Co-ral and three rats were sacrificed at each of 10 time intervals after treatment. Blood samples were removed from the heart at the time of sacrifice, and heparin was added immediately as an anti-coagulant. Co-ral residues remaining on the hide were washed off with 10 ml. of acetone per animal with the acid on cotton swabs.

Table I. Distribution and Excretion of Co-ral Applied Dermally to Male White Rats at 45 Mg. per Kg.[a]

		Days after Treatment									
		0.16	0.25	0.50	1	2	4	7	14	21	28
		% Applied Co-ral Equivalents Recovered									
From skin swabs		26	1.8	2.8	1.5	2.0	0.7	0.1	<0.01	<0.01	<0.01
From skin at application site		38	56	65	74	57	29	9.5	2.5	1.1	0.8
Excreted in urine and feces		0.7	1.4	2.4	4.0	7.5	11	16	22	24	25
In all tissues analyzed other than skin		0.9	0.4	0.4	0.6	1.1	1.2	1.3	0.5	0.2	0.1
		P.P.M. Co-ral Equivalents in Tissues									
Skin at applic. site	$CHCl_3$-soluble	235	276	186	222	154	94	34	5.7	3.0	2.5
	H_2O-soluble	32	22	16	11	12	8.8	1.7	0.5	0.2	0.2
	Unextractable	237	324	457	473	408	189	64	16	11	6.2
Fat	$CHCl_3$-soluble	1.8	5.5	2.8	2.4	2.0	1.4	..	..	..	..
	H_2O-soluble	0.3	0.1	0.4	0.4	0.3	0.2	2.0[b]	1.0[b]	0.04[b]	0.08[b]
	Unextractable	1.6	1.6	1.6	..	1.7	1.4	..	..	..	..
Liver	$CHCl_3$-soluble	0.4	0.4	0.2	0.4	0.5	0.2	0.4	<0.04	..	..
	H_2O-soluble	1.3	0.9	0.4	0.7	1.8	1.0	1.7	0.8	1.4[b]	0.6[b]
	Unextractable	5.3	1.4	2.7	4.2	7.2	7.4	8.4	3.7	..	..
Kidney	[b]	2.0	1.0	1.2	2.9	3.8	3.9	1.7	1.2	0.4	0.1
Brain	[b]	0.4	0.3	0.2	0.3	0.6	0.7	0.8	0.7	0.4	0.3
Spleen	[b]	1.0	0.4	2.1	1.3	1.2	2.0	3.6	1.1	0.5	0.3
Heart	[b]	0.6	0.1	0.2	0.6	0.9	1.6	1.5	0.6	0.2	0.1
Blood	[b]	0.9	0.3	0.5	0.6	0.9	1.1	1.1	0.3	0.1	<0.08

[a] Average of three replicates. [b] Not fractionated.

The total radioactivity in the tissues was expressed as parts per million of Co-ral equivalents (Table I).

Fractionation Procedure for Co-ral and Metabolites. The fractionation procedure for Co-ral and metabolites was based on the ability to extract 100% of Co-ral and its oxygen analog into chloroform from neutral aqueous solutions without recovering any of the hydrolysis products in the organic layer. The various rat tissues were macerated with 50 ml. of chloroform and 25 ml. of water in a Waring Blendor. The homogenate was then centrifuged, the chloroform-protein phase was filtered through Celite, and the chloroform was dried over anhydrous sodium sulfate. The chloroform-insolubles remaining on the Celite were homogenized with 50 ml. of acetone, filtered through sodium sulfate, and evaporated, and the residue was repartitioned between chloroform and water—the chloroform being added to the original chloroform extract. The three resulting fractions were designated as chloroform-soluble, water-soluble, and unextractable residue.

Distribution of Co-ral and Metabolites in Tissues. Following dermal administration of radioactive Co-ral to rats, all of the tissues analyzed showed an initial peak of radioactivity between 4 and 6 hours after treatment then a drop before a subsequent uniform increase to a second peak after 4 to 7 days (Table I). This may have resulted from some of the Co-ral penetrating with the solvent in the emulsion and the remainder entering by a slower partitioning process. The total tissues analyzed accounted for approximately 20% of the rats' weight.

Rate of Excretion and Characterization of Excretion Products. The excretion of Co-ral following administration to the rat is shown in Figure 2. Following dermal administration two thirds of the amount excreted appeared in the urine within 28 days, compared with four fifths within 14 days of oral or 18 days of subcutaneous administration.

Four Co-ral metabolites were detected in the rat urine using ion exchange chromatography. During the first 96 hours after oral treatment, 50 to 80% of the radioactivity excreted was as diethyl phosphorothioic acid. Phosphoric and diethyl phosphoric acids were excreted in approximately equal proportions (10 to 20% each). A fourth metabolite, the de-ethylated derivative of Co-ral and/or its oxygen analog, was excreted in amounts of 5 to 30%—the larger proportionate amounts appeared immediately following administration of the insecticide. Subcutaneous administration resulted in three metabolites. Approximately twice the proportionate amount of diethyl phosphoric acid was excreted as compared to the oral treatment while no phosphoric acid was found. Diethyl phosphorothioic acid was found in roughly equal proportion to the oral administration and de-ethylated derivatives of Co-ral and/or its oxygen analog were found at low levels (less than 10%) and then only in the early urine samples following administration. Dermal treatment resulted in approximately equal proportions of phosphoric, diethyl phosphoric, and diethyl phosphorothioic acids while none of the de-ethylated derivatives could be identified in the 48- to 72-hour sample.

Fractionation of the feces showed 17% of the total radioactivity to chromatograph as Co-ral or its oxygen analog when the rats were treated subcutaneously. Oral and dermal administration resulted in 50 to 60% of the total radioactivity in the feces fractionating as Co-ral or its oxygen analog. Feces samples fractionated contained the highest parts per million of Co-ral equivalents.

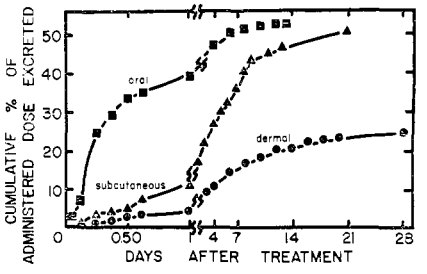


Figure 2. Excretion of Co-ral and metabolites in rat urine and feces following subcutaneous, dermal, and oral administration of Co-ral at 40, 45, and 50 mg. per kg., respectively

The oxygen analog administered orally at 20 mg. per kg. resulted in 50% of the excreted radioactivity as phosphoric acid, 10% as diethyl phosphoric acid, and 40% as the de-ethylated derivative of the oxygen analog—composite urine sample of the first 24 hours.

Co-ral Distribution and Metabolism in a Cow and Goat Dermal Administration and Animal Handling. A 644-kg. lactating Holstein cow was treated with 40 mg. of Co-ral per kg. of body weight corrected for losses in administration. An 0.75% Co-ral emulsion was prepared by dissolving 28.4 grams of radioactive Co-ral (160 c.p.m. per γ) in 142 ml. of xylene containing 18.9 ml. of Triton X-155 and adding this emulsifiable concentrate to 1 gallon of warm water. This emulsion was sprayed onto the cow with a 3-gallon hand sprayer at 20 pounds-per-square-inch pressure with

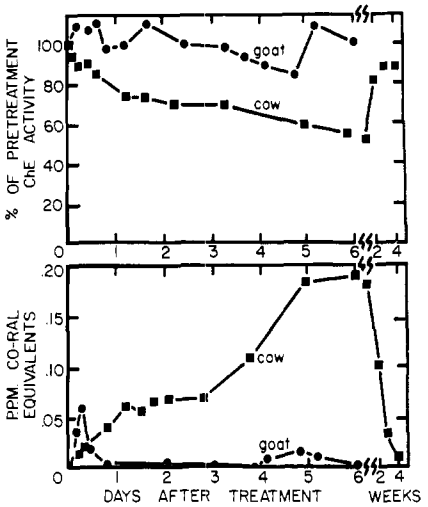


Figure 3. Co-ral metabolites in blood and depression of blood cholinesterase activity after dermal administration of Co-ral to a cow and a goat at 40 and 30 mg. per kg., respectively

Table II. Co-ral and Metabolite Levels after Dermal Administration of Co-ral[a]

P.P.M.	Both	Cow[b]	Goat[c]
<0.2	Bone marrow, large intestine	Bile, fat, thyroid, bladder	Rib bone, brain, mammary, parotid and submaxillary glands, heart, lymph nodes (except axillary) muscle, pancreas, spinal cord, spleen, omasum, abomasum, uterus
0.2 to 0.5	Adrenal, small intestine, lung, reticulum, rumen, tongue	Brain, gall bladder parotid and submaxillary glands, heart, lymph nodes, muscle, spinal cord, spleen, omasum, abomasum, uterus	Fat, thyroid
>0.5		Rib bone (9.60), mammary gland (0.52), kidney (0.96), liver (1.22), axillary lymph node (0.81)	Bile (0.93), gall bladder (0.59), kidney (0.90), liver (3.74), bladder (0.59)

[a] Results are averages of three replicates. Parenthetical figures are parts per million. [b] Dosage, 40 mg./kg. Tested after 8 weeks. [c] Dosage, 30 mg./kg. Tested after 6 days.

care to minimize loss from drift and runoff. The head, neck, and udder were covered with plastic sheeting to prevent their contamination during spraying; precautions were not taken against licking. The cow was held in a 12 × 12 foot isolated pen except during milking and bleeding. Milk samples were taken with a machine after thoroughly washing the udder with 95% ethyl alcohol.

A 54-kg. lactating goat was treated with 30 mg. of Co-ral per kg. of body weight by dissolving the radio-labeled insecticide (225 c.p.m. per γ) in 50 ml. of xylene and painting this xylene solution on the back of the recently clipped goat. No loss of Co-ral was encountered with this method of application. Throughout the course of the experiment, the goat was held in a metabolism stanchion, which allowed quantitative collection of urine and feces and prevented the goat from licking the site of application. Hand milking was utilized after thoroughly washing the udder.

Fractionation Procedures for Co-ral and Metabolites. All tissues, except fat, were extracted first with acetone and then with benzene. The solvent order was reversed with the fat. The radioactivity in the residual dry powder was considered as unextractable. The acetone and benzene extracts were combined, evaporated to dryness, re-dissolved in Skellysolve B and partitioned with an equal volume of water to remove hydrolysis products. The Skellysolve B phase was extracted with an equal volume of acetonitrile to recover Co-ral and its oxygen analog but very little fat in the acetonitrile. Co-ral and its oxygen analog partitioned 97+ % into acetonitrile from Skellysolve B. The acetonitrile was then evaporated and the radioactive materials chromatographed on an alumina column with carrier Co-ral and oxygen analog. The radioactivity eluting with benzene was considered to be Co-ral and that with chloroform was considered to be oxygen analog. However, the radioactivity recovered with benzene and chloroform from the alumina column might also contain some phospholipides. When known amounts of radioactive Co-ral were added in Skellysolve B to liver, fat, and muscle samples, recoveries with the procedure were 92, 91, and 94%, respectively.

Feces samples were extracted with acetone and then water. The acetone was evaporated to dryness, the water extract was added to it, and the combination was extracted with chloroform. The chloroform was evaporated to dryness and the radioactivity was chromatographed with carrier Co-ral and oxygen analog to determine the nature of the extracted materials.

Urine samples were extracted with an equal volume of chloroform—the radioactivity in this first chloroform extract representing intact Co-ral and its oxygen analog. The water phase was adjusted to pH 1 with hydrochloric acid and held for 30 minutes at room temperature after which time it was partitioned with an equal volume of chloroform. The water phase was then adjusted to pH 7 and re-partitioned with the same chloroform. The radioactivity in the second chloroform extract represented the Co-ral and its oxygen analog which was originally in a water-soluble form, (this water-soluble form may have resulted from interaction with the urine proteins or emulsification, but more probably resulted from the opening of the pyrone ring).

One hundred milliliters of fresh whole milk were extracted with 100 ml. of 95% ethyl alcohol and 20 ml. of Skellysolve B. The Skellysolve B was separated by centrifugation and extracted with an equal volume of acetonitrile, and the acetonitrile-soluble radioactive material was chromatographed with carrier nonlabeled Co-ral and its oxygen analog. The defatted milk ethyl alcohol phase was thoroughly mixed and analyzed for radioactivity which was due to hydrolysis products and other forms of unextractable material. When Co-ral was added to milk in Skellysolve B, a 99% recovery was obtained by this procedure.

The total parts per million of Co-ral and derivatives in heparinized venous blood were calculated from the radioactivity. Fifty milliliters of blood were then diluted to 250 ml. with water and extracted with an equal volume of Skellysolve B. The cholinesterase activity of the heparinized whole blood was determined manometrically using 0.20 ml. of cow blood and 0.40 ml. of goat blood (*5*). The results are expressed as per cent of pretreatment values determined from the same animal (Figure 3).

All radioactive samples were counted for sufficiently long periods to ensure statistical accuracy to at least ±10%. Errors of up to 20% are also possible in the radioactive residue results from inability to compensate precisely for self-absorption with the varying types of samples counted, particularly where large amounts of fats were present in the samples. These factors may partially account for the failure of the tissue fractionation results (Table III) to total the same as the results from direct radioactive counts on the tissues (Table II). Another factor involved is the omission of water-soluble metabolites from Table III because of the insensitivity of the method for counting this fraction.

Distribution of Co-ral and Metabolites in Tissues. The distribution of total radioactive Co-ral and metabolites in the tissues of the cow and goat is given in Table II. Fractionation of various cow and goat tissues are shown in Table III. Unextractable residues were quite significant with all the tissues, while the hydrolysis products recovered with the

Table III. P.P.M. of Co-ral[a] and Metabolites after Dermal Administration of Co-ral[a]

Sample[b]	Acetone-Benzene Extract: Skellysolve B solubles[c]	Acetone-Benzene Extract: Acetonitrile solubles	Unextractable Residue	% P^{32} in Acetonitrile Chromatographing as Co-ral[d]
		Cow[e]		
Brain	0.13 ± 0.05	0.012 ± 0.003	0.10 ± 0.03	..
Fat				
Intestinal	<0.16	0.056 ± 0.009	0.028 ± 0.021	36
Omental	<0.16	0.049 ± 0.002	0.012 ± 0.005	23
Perirenal	<0.16	0.045 ± 0.042	0.058 ± 0.051	0
Heart	0.11 ± 0.03	0.015 ± 0.005	0.22 ± 0.01	..
Kidney	0.42 ± 0.03	0.040 ± 0.010	0.34 ± 0.04	35
Liver	0.23 ± 0.05	0.021 ± 0.002	0.67 ± 0.10	28
Lung	0.12 ± 0.01	0.014 ± 0.004	0.22 ± 0.03	..
Muscle				
Back	0.045 ± 0.005	0.029 ± 0.008	0.18 ± 0.03	45
Hind leg	0.049 ± 0.008	0.043 ± 0.021	0.18 ± 0.02	60
Loin	0.091 ± 0.010	0.044 ± 0.005	0.22 ± 0.03	44
Spleen	0.081 ± 0.021	0.017 ± 0.006	0.15 ± 0.02	36
		Goat[f]		
Brain	0.005 ± 0.002	0.008 ± 0.001	0.014 ± 0.009	100
Fat				
Intestinal	<0.025	0.14 ± 0.02	0.003 ± 0.002	97
Omental	<0.025	0.18 ± 0.01	0.016 ± 0.005	98
Perirenal	<0.025	0.16 ± 0.01	0.006 ± 0.002	96
Subcutaneous	<0.025	0.12 ± 0.02	0.025 ± 0.006	96
Heart	0.004 ± 0.002	0.022 ± 0.004	0.055 ± 0.003	90
Kidney	0.64 ± 0.02	0.24 ± 0.01	0.20 ± 0.02	100
Liver	<0.004	0.13 ± 0.01	1.94 ± 0.20	100
Lung	0.008 ± 0.001	0.008 ± 0.001	0.22 ± 0.05	100
Muscle				
Back	<0.004	0.008 ± 0.001	0.011 ± 0.003	100
Hind leg	<0.004	0.011 ± 0.003	0.029 ± 0.015	100
Loin	<0.004	0.009 ± 0.001	0.024 ± 0.022	100
Spleen	0.005 ± 0.002	0.004 ± 0.002	0.087 ± 0.017	100

[a] The water-soluble metabolites are not tabulated as the analysis method was low in sensitivity and no radioactivity was detected in this fraction (except in goat liver and kidney where values were 1.22 and 0.7 p.p.m. Co-ral equivalents, respectively). [b] Three replicates per sample except with brain where there were only two replicates. Variation expressed as average ± standard deviation. [c] Skellysolve B solubles represent the radioactivity in the Skellysolve B phase from the Skellysolve B-acetonitrile partitioning plus the radioactivity in the acetonitrile phase which was eluted from the alumina columns with Skellysolve B. [d] The remaining radioactivity was eluted from the alumina columns with chloroform in a similar manner to the oxygen analog of Co-ral. [e] Dosage, 40 mg./kg. Tested after 8 weeks. [f] Dosage, 30 mg./kg. Tested after 6 days.

described procedure were below the sensitivity limit of the method. Acetonitrile-soluble fractions were chromatographed on alumina columns. Many of the tissues, particularly the fat, contained a considerable amount of radioactivity chromatographing like the oxygen analog. For the goat, the kidney was the only tissue which contained appreciable Skellysolve B-soluble radioactivity, and hydrolysis products were highest in the kidney and liver. Almost all of the acetonitrile-soluble radioactivity chromatographed on alumina columns as anticipated.

Blood Cholinesterase Levels and Co-ral Equivalents Present in the Blood. For the cow, the peak blood level of total radioactivity (0.20 p.p.m. Co-ral equivalents) occurred at 6 days, and the peak level of Co-ral or its oxygen analog appeared at 5 days after treatment (0.015 p.p.m. Co-ral equivalents of which less than 10% chromatographed as oxygen analog) (Figure 3). The whole blood cholinesterase level showed a continual drop until the seventh day after which time recovery occurred to nearly pretreatment activity by the end of 2 weeks. The inhibition of cholinesterase is probably due to the presence of the oxygen analog of Co-ral, as in all toxic thionophosphate studies so far the parent material is a weak anticholinesterase, and is converted in vivo to the P=O form which is a potent anticholinesterase.

The goat showed two peaks in total radioactivity in the blood, the first at 6 hours and the second at 5 days after treatment (Figure 3). This may have resulted from some of the Co-ral penetrating with the solvent and the remainder entering by a slower partitioning process. Fractionation of the blood showed the levels of Co-ral or its oxygen analog to be less than 0.004 p.p.m. throughout the experiment. The cholinesterase levels were somewhat variable, but little if any inhibition was noted.

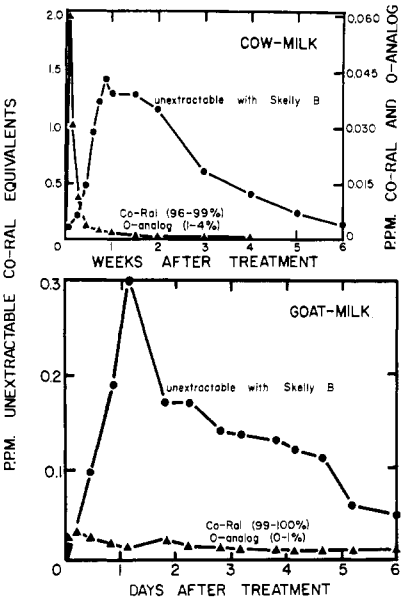


Figure 4. Co-ral equivalents in the milk of a cow and a goat following dermal administration of Co-ral at 40 and 30 mg. per kg., respectively

No symptoms of organophosphate poisoning were noted with either the cow or goat and no pathological abnormalities attributable to the Co-ral were found on sacrificing the animals.

Milk Residues. The residues in cow and goat following dermal application are shown in Figure 4.

Rate of Excretion and Characterization of the Excretory Products. With the cow, excretion in both the urine and feces was important in eliminating Co-ral and its degradation products (Figure 5). Most of the radioactivity appearing in the urine was as hydrolysis products. The peak excretion of unhydrolyzed insecticide appeared in the 0- to 6-hour urine where 2.1% of the total radioactivity fractionated as unhydrolyzed Co-ral and 0.9% as oxygen analog. The proportion of unhydrolyzed material in the urine declined steadily so that by the end of 1 week 99.9% of the radioactivity in the urine appeared as hydrolysis products. After extracting separate samples of the 4-, 6-, 12-, and 48-hour urine with chloroform, the aqueous phase was adjusted to pH 1, then re-adjusted to 7 and re-extracted with chloroform as described under fractionation procedures. This manipulation allowed a further recovery of 3% of the total radioactivity in chloroform of which 5% chromatographed as Co-ral and 95% as oxygen analog. Ion exchange chromatography of urine samples taken at 0.25, 1, 4, and 7 days after treatment indicated approximately 36% phosphoric, 17% diethyl phosphoric, and 29% diethyl phosphorothioic acids with 18% as the de-ethylated derivative of Co-ral or its oxygen

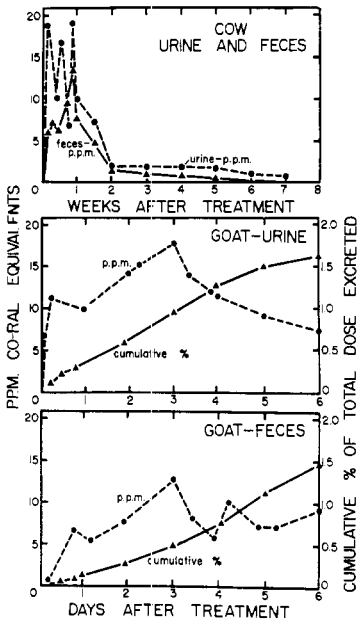


Figure 5. Co-ral metabolites in the urine and feces of a cow and a goat following dermal administration of Co-ral at 40 and 30 mg. per kg., respectively

analog. Fractionation of the 6-day feces sample showed that 32% of the total radioactivity was Co-ral and 6% was oxygen analog.

The p.p.m. and cumulative excretion data for the goat are presented in Figure 5. At 3 days when the level of excreted radioactivity in the urine was at a maximum, only 1.2% fractionated as Co-ral and 1.8% as the oxygen analog. Re-extraction of the aqueous phase of the 0- to 72-hour urine after acidification recovered an additional 1.0% of the total radioactivity of which 30% fractionated as Co-ral and 70% as oxygen analog. Ion exchange chromatography of the 3- and 4-day urine samples showed 25% phosphoric, 18% diethyl phosphoric, and 37% diethyl phosphorothioic acids with 20% as the de-ethylated derivative of Co-ral or its oxygen analog. Fractionation of the 6-day feces sample yielded 31% of the total radioactivity as Co-ral and 1.6% as oxygen analog.

Persistence of Dermal Residues. Four uniformly distributed samples of 15 grams of hide each were selected from the treated area of the goat and similar representative samples were selected from the untreated areas, 6 days after treatment. With the cow, 20-gram hide samples were selected from 15 evenly distributed areas of the treated portion 8 weeks after treatment. Each hide sample was soaked in 100 ml. of acetone for 24 hours and then rinsed with 50 ml. of fresh acetone. The radioactivity extracted with the acetone and remaining in the hide residue was determined.

The cow hide averaged 12 p.p.m. of extractable Co-ral equivalents (range of 2.1 to 24 p.p.m.) and 1.5 p.p.m. (range of 0.8 to 2.2 p.p.m.) of unextractable residue. Excluded from these averages was the front shoulder region which had approximately 40 times the radioactivity present on the rest of the hide.

The treated area of the goat had an average extractable Co-ral equivalent of 4070 p.p.m. (range of 3600 to 4400 p.p.m.) and an unextractable residue of 336 p.p.m. (range of 290 to 430 p.p.m.). The untreated area of the goat had an extractable residue of 237 p.p.m. (range of 217 to 261 p.p.m.) and an unextractable residue of 18 p.p.m. (range of 15 to 20 p.p.m.).

Discussion

Co-ral and its oxygen analog differ from most dialkyl aryl phosphorothioates and phosphates as they contain a pyrone ring which is readily opened in the presence of dilute alkali (pH 10 to 12) without hydrolysis of the aryl phosphate bond. The ease of opening this ring was identical with the diethyl phosphorothioate and diethyl phosphate derivatives. Acidification of these water-soluble salts of the substituted-coumarinic acids effected a complete reversion to the original compound. With selective alkaline conditions both the phosphate and phosphorothioate were cleaved at either the phosphorus-oxygen-ethyl or phosphorus-oxygen-coumarinyl groupings. In this latter respect, Co-ral and its oxygen analog are similar to *O,O*-dimethyl *O*-(2,4,5-trichlorophenyl) phosphorothioate and phosphate (*13*) and several other dimethyl and diethyl aryl phosphorothioates (*14*).

When administered to mammals, Co-ral and its oxygen analog underwent the same metabolic pathway as certain other dialkyl aryl phosphates and phosphorothioates (*13, 14*) except that the complete degradation of the compounds to phosphoric acid appeared to occur more rapidly than with other phosphate insecticides which have been studied. Co-ral was more susceptible to cleavage of the phosphorus-oxygen-ethyl group in vivo than either Diazinon or parathion (*14*). This formation of phosphoric acid was further indicated from the high levels of radioactive phosphorus that appeared in the bones of treated animals. The pH of the cow and goat urine was 8.0 and 8.5, respectively, conditions under which 13 and 38% of both Co-ral and its oxygen analog would appear with the open lactone ring. Fractionation of the urine from treated animals indicated that a portion of the radioactivity in the cow and goat urine partitioned as if the lactone ring were open but the rest of the molecule intact. The metabolic products of Co-ral are summarized in Figure 6.

It is not possible to compare the excretion rate and tissue residues in the rats, cow, and goat because of differences in application method, time before sacrifice, and techniques of extraction. With both the rats and goat a small portion of the Co-ral was very rapidly absorbed through the skin, and then the rate of penetration greatly diminished as if the solvent had swept some of the material in rapidly, and the remainder entered by a slower partitioning process.

Extreme difficulties were encountered in using the phosphorus-32 label to evaluate the residual persistence of Co-ral. An appreciable proportion of the total radioactivity was not extracted from the tissues by the methods used and the chemical forms of these radioactive compounds is now known. The phosphorus-32 from Co-ral formed phosphoric-32 acid which entered into the metabolic pool of the cow and probably formed phosphoproteins and phospholipides. The phosphoproteins may have contributed to the unextractable radioactivity and certain of the phospholipides may have appeared in the same fractions as Co-ral and the oxygen analog. These factors must be considered in evaluating the residue results presented.

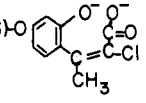
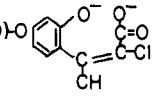
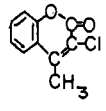
METABOLITE	SOURCE
$(C_2H_5O)_2P(S)$-OX	TISSUES, MILK, BLOOD, URINE, FECES
$(C_2H_5O)_2P(O)$-OX	TISSUES, MILK, URINE, FECES
C_2H_5O, HO, P(S)-OX	URINE
$(C_2H_5O)_2P(S)OH$	URINE
$(C_2H_5O)_2P(O)OH$	URINE
H_3PO_4	URINE
$(C_2H_5O)(HO)P(O)OH$	(SOME EVIDENCE FOR PRESENCE IN URINE)
$(C_2H_5O)_2P(S)$-O- 	URINE (?)
$(C_2H_5O)_2P(O)$-O- 	URINE (?)
C_2H_5O, HO, P(O)-OX	URINE
NOTE X= 	

Figure 6. Co-ral metabolites characterized with source given for each

Acknowledgment

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ANIMAL METABOLISM OF INSECTICIDES

Bovine Metabolism of Organophosphorus Insecticides. Metabolism and Residues Associated with Oral Administration of Dimethoate to Rats and Three Lactating Cows

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Dimethoate is active as a systemic insecticide for cattle. Three lactating cows were treated orally with the radioactive compound and analysis of blood, tissues, excreta, and milk showed Dimethoate to be rapidly metabolized and excreted. Twelve days after treatment, the insecticide was found in trace amounts only in the cow tissues. Hydrolysis of Dimethoate by rats and cows occurred initially at the methyl-phosphate, phosphate-sulfur, sulfur-carbon, and particularly at the carbonyl-nitrogen bonds. Phosphorothioate oxidation occurred with certain of the hydrolysis products and was assumed to occur also with Dimethoate.

DIMETHOATE or Am. Cyanamid 12,880 [*O,O*-dimethyl *S*-(*N*-methylcarbamoylmethyl)phosphorodithioate] has shown promise as a chemotherapeutic agent for the control of cattle grubs. Oral administration to calves of 10 to 40 mg. per kg. yields excellent grub control (*5*). When tested as an animal systemic against *Aedes* mosquitoes, it gave excellent control during the first 24 hours, but decreased rapidly thereafter (*4*). This study concerns the residues and metabolites associated with the proposed use of Dimethoate as an animal systemic insecticide.

Methods and Results

Chemical Studies

Synthesis and Characterization of Radioactive Dimethoate Derivatives. Radioactive *O,O*-dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorodithioate was prepared from phosphorus-32 pentasulfide obtained by neutron irradiation (Atomic Energy Commission, Oak Ridge, Tenn.) or by isotopic exchange with phosphoric-32 acid (*1*). Radioactive *O,O*-dimethyl phosphorodithioic acid was then prepared (*9*) and neutralized with potassium hydroxide to yield the potassium salt in aqueous solution. An amount of *N*-methyl α-chloroacetamide equimolar to the potassium *O,O*-dimethyl phosphorodithioate was dissolved in water, layered with chloroform, and the two-phase system stirred rapidly and refluxed at 85° C. as the potassium *O,O*-dimethyl phosphorodithioate solution was added dropwise over a 30-minute period (*14*). After an additional 15 minutes of refluxing, the chloroform layer was separated, and the aqueous phase extracted three times with an equal volume of chloroform. The product formed in 60% yield had a specific activity of about 5 mc. per gram and it was purified on a silica gel column (Figure 1). *O,O,S-*