Table III. Statistical Analysis of Precision of Analytical Methods

Analytical Method	Mean, Y	Variance, S^2	Standard Deviation, s	Coefficient of Variation, %
Ca + Mg	8.161	0.000308	0.01755	0.22
Mg direct	0.4416	0.00000445	0.00211	0.48
Ca indirect	0.9776	0.0000130	0.00360	0.37

extraction rather than by an exhaustive leaching. Four samples of 20 grams of soil were weighed, soaked in 100 ml. of 1*N* neutral ammonium acetate solution for 1 hour, agitated on a shaker for 30 minutes, and filtered through Whatman No. 42 filter paper. Aliquots of 10 ml. of this extract, each representing 2 grams of soil, were suitable for either direct determination of magnesium or the combined calcium and magnesium determination by the titration procedures previously described. The calcium equivalent of EDTA was obtained by subtracting the magnesium equivalent of EDTA from that of calcium plus magnesium.

The precipitated manganese ferrocyanide was slight and caused no difficulty in titrations, although Cisne soil is relatively high in easily soluble manganese. With the weight of soil sample and size of aliquots used in these experiments, interference from exchangeable manganese in amounts less than 1 meq. per 100 grams of soil is eliminated simply by the addition of ferrocyanide. If the amounts of manganese are higher, the precipitate should be removed by filtration to avoid difficulty in detection of the end point.

Data in Table II are from four aliquots of extracts of each of the four samples of soil and show excellent agreement within samples as well as among samples.

Statistical analyses of these data are shown in Table III. Coefficients of variation were 0.22, 0.48, and 0.37%, respectively, for the calcium plus magnesium titration, magnesium titration, and calcium determination by difference. Validity of these statistical statements is limited to the operator and conditions of these experiments, but good agreement is indicated.

Acknowledgment

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INSECTICIDE ASSAY

Assay of Co-Ral in Technical Material and Formulated Products

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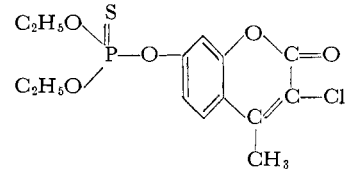
Two analytical methods for the determination of Co-Ral in technical material and formulated products are described. These involve alkaline hydrolysis and ultraviolet absorption spectrophotometry. The former method gives the more precise results. However, the latter allows for easy correction for 3-chloro-4-methylumbelliferone in the material and is less time-consuming than the former. Both methods have been used successfully for routine control purposes.

Co-Ral, formerly known as Bayer 21/199, is a commercial insecticide for the control of various insect pests of domestic animals. The compound is *O,O* - diethyl - *O* - 3 - chloro - 4 - methyl - 2-oxo-2*H*-1-benzopyran-7-yl phosphorothioate. The structural formula is



For the analysis of both technical material and formulations, a reproducible method of assay was required. The literature contains numerous references to analytical methods for coumarin derivatives, exemplified by the work of Ensminger (*2*). However, most of these methods have been designed for trace analysis and were not considered sufficiently accurate for assay purposes.

A preliminary examination of possible methods of analysis of the above compound indicated alkaline hydrolysis and ultraviolet absorption to be the most promising techniques for further study. Both of these approaches have been investigated and both have yielded satisfactory analytical procedures.

From a consideration of the structure of Co-Ral, hydrolysis was expected to form diethylphosphorothioic acid from saponification of the ester grouping and hydrochloric acid from the chlorine in the lactone ring. Ring opening in alkaline solution should form a carboxylic acid which should also be titratable. In addition to this, the above reactions would form two weakly acid phenolic groups. If ring closure to form a benzopyran ring occurs (*9*), only the remaining phenolic group would be titratable. Thus one might expect to obtain four or possibly five equivalents of acid on alkaline hydrolysis of Co-Ral.

The aromatic structure of Co-Ral suggests the use of ultraviolet absorption as an assay method. Coumarin derivatives (*2*) are known to be strong absorbers in the ultraviolet. In addition to this, Hensel *et al.* (*5*) have described an ultraviolet absorption method for the unchlorinated derivative of Co-Ral (Potasan).

A fluorescence method for determination of Co-Ral residues in animal tissues has been published recently (*1*).

Experimental

Hydrolysis of Co-Ral. The alkaline hydrolysis of Co-Ral was investigated in both water and isopropyl alcohol. Samples of recrystallized Co-Ral were hydrolyzed by refluxing for 2 hours in 1*N* aqueous and 1*N* isopropyl alcoholic

alkali. In both cases, the hydrolyzates were back-titrated with 0.5*N* hydrochloric acid using a Precision Scientific automatic titrator.

Figure 1 shows that the titration curves obtained are different, depending on whether the hydrolysis is carried out in water or in isopropyl alcohol. The aqueous titration curve has four inflections—at pH 11.0, 7.5, 5.5, and 2.6. These inflection points correspond respectively to 4.5, 3, 2.5, and 2 equivalents of acid per mole of Co-Ral. The alcoholic titration curve has only three inflections at pH 11.6, 7.5, and 3.0. These points correspond to 4, 3, and 2 equivalents of acid formed per mole of Co-Ral. The break which occurred at pH 5.5 following aqueous hydrolysis is not present in the titration curve for the alcoholic hydrolysis medium.

The appearance of the hydrolyzed solutions was markedly different, depending on whether water or isopropyl alcohol was used as solvent. Co-Ral is initially partially insoluble in the aqueous alkali, but after a few minutes of boiling, the solution becomes clear and remains so. After hydrolysis, the solution is a purple color and it undergoes a series of color changes during titration, turning gray-blue at pH 10.0, brown at pH 7.5, and orange-pink at pH 3.5. Below pH 4.0 the solution becomes turbid and a white crystalline precipitate forms on standing. Co-Ral is readily soluble in the alcoholic reagent, but insoluble material is formed during hydrolysis. This material dissolves readily in the water added before titration. At this point, the solution is dark green in color and highly fluorescent. During titration, this solution changes to brown at pH 11.5, and faintly pink at pH 7.0. Below pH 7.0 some turbidity is noted, but a definite crystalline precipitate does not form.

In both cases, the only inflection which is satisfactory for quantitative work is that which occurs at pH 7.5. In both the aqueous and alcoholic media, this corresponds to the formation of three moles of acid per mole of Co-Ral. It is assumed that these are diethyl phosphorothioic acid, hydrochloric acid, and a carboxylic acid formed by opening the lactone ring.

The effects of hydrolysis time and strength of alkali were investigated with both media using the end point at pH 7.5. The effect of hydrolysis time is shown in Figure 2. In both cases, no further hydrolysis occurs after 2 hours. The reaction is completed more rapidly in the alcoholic than in the aqueous medium.

The effect of dilution of the hydrolysis solution was tested, using both media. Increment amounts of water or isopropyl alcohol were added to the appropriate solutions before refluxing for 2 hours. In both cases, dilution to an alkali concentration of 0.5*N* decreased the amount of hydrolysis in 2 hours to 97 to 98% of the value obtained with 1.0*N* alkali. Further dilution of the alkali decreased the amount of hydrolysis markedly.

Consideration of the more definite stoichiometric relationship, more rapid reaction, and somewhat better end point indicated the choice of isopropyl alcohol over water as solvent for hydrolysis of this compound. Accordingly this was adopted for quantitative work.

Mechanism of Hydrolysis. According to Wawzonek (*10*) the initial action of alkali on coumarin structures is to open the lactone ring to form a salt of coumarinic acid. Acidification regenerates the original coumarin. That this happens with Co-Ral was demonstrated by shortening the hydrolysis time to a minimum. The solution was cooled as soon as the solid dissolved in 1*N* isopropyl alcoholic potassium hydroxide—about 2 minutes. Addition of water resulted in a pale yellow, slightly fluorescent solution. Titration of the hydrolyzate gave an inflection at pH 11.2. Precipitation of a white crystalline material began at pH 9. Each additional drop of acid was followed by a sharp drop in pH and then a slow return to pH 9 to 10. This continued until all of the alkali originally present was neutralized, indicating that no base had been consumed by the Co-Ral. The precipitate which formed on acidification was separated and identified by melting point and infrared absorption as Co-Ral.

This shows conclusively that the first action of alkali is the opening of the lactone ring (Reaction I).

On heating with alkali, three additional reactions can be visualized. The phosphate ester linkage would be split to form the potassium salt of diethylphosphorothionate. Reactions of this type have been described for many organic phosphate pesticides (*8*). The

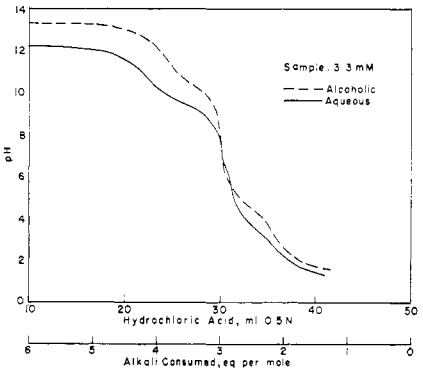


Figure 1. Titration of aqueous and alcoholic Co-Ral hydrolyzates with hydrochloric acid

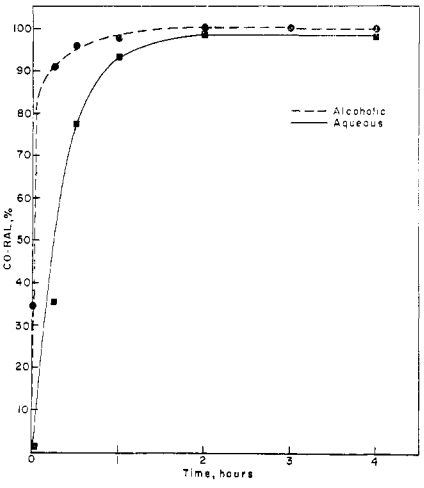
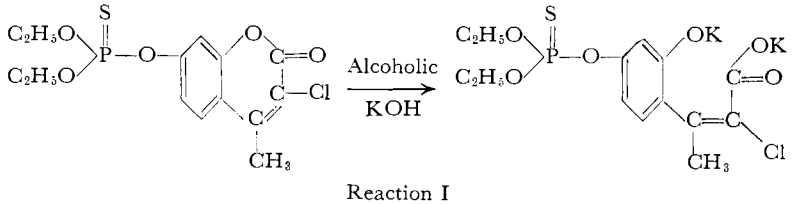
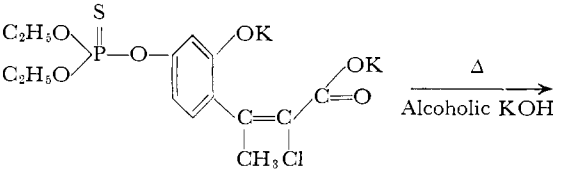


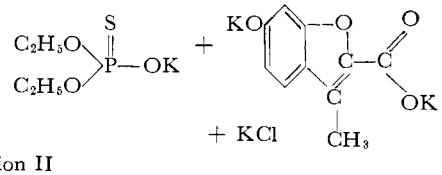
Figure 2. The effect of time on hydrolysis of Co-Ral

secondary phosphate esters are stable to alkaline hydrolysis (*7*) and, therefore, would not undergo further decomposition. The isomerization of the *cis*-coumarinic acid to a *trans*-coumaric acid has been described by Fries *et al.* (*3*, *4*). This would be expected to be followed by ring closure to form a benzofuran ring as originally described by Perkin (*9*).



Reaction I





Reaction II

The actual order in which the above reactions occur is not known, but the final products of hydrolysis are shown in Reaction II.

These reactions would explain completely the observations made on the hydrolysis of Co-Ral with alcoholic potassium hydroxide.

Ultraviolet Absorption. The principal interferences expected in an ultraviolet absorption method for Co-Ral would be chloromethylumbelliferone or Potasan (the unchlorinated compound corresponding to Co-Ral). The former compound could result either from incomplete reaction during manufacture or from hydrolysis as a result of decomposition of formulated products during storage. It was expected that the ultraviolet spectra of Co-Ral and chloromethylumbelliferone would overlap too closely to allow for the quantitative determination of one in the presence of the other. However, the free phenolic hydroxyl group in the latter compound offered the possibility of forming the phenolate in alkaline solution. Formation of phenolates from *m*-substituted phenols is known to cause a bathochromic shift (*6*). In view of this, the ultraviolet absorption spectra of Co-Ral and chloromethylumbelliferone were determined in both the presence and absence of sodium carbonate.

Solutions containing 10 γ per ml. of Co-Ral and 5 γ per ml. of chloromethylumbelliferone in absolute methanol were used in this experiment. The ultraviolet absorption spectra were determined in a Beckman DU spectrophotometer using a 1-cm. cell. The absorption spectra are shown in Figure 3. The results show a double peak for Co-Ral with maxima at 290 and 315 mμ. Chloromethylumbelliferone has an absorption maximum at 330 mμ. Direct estimation of one compound in the presence of the other would be possible by setting up simultaneous equations and measuring the absorbance at 290 and 330 mμ. As this would not be a very accurate procedure, the effect of sodium carbonate on the absorbance of both compounds was determined. In this experiment, solutions containing 10 γ per ml. of Co-Ral and 5 γ per ml. of chloromethylumbelliferone were prepared in 50% aqueous methanol. The solutions contained 0.5% of sodium carbonate. The spectrophotometric results are shown in Figure 3. Sodium carbonate causes a bathochromic shift in the absorbance of chloromethylumbelliferone from 330 to 380 mμ. Under the same conditions, Co-Ral is practically nonabsorbing at 380 mμ. The specific extinction values for Co-Ral and chloromethylumbelliferone in methanol at 290 mμ and for chloromethylumbelliferone in a solution of methanol, water, and sodium carbonate are shown in Table I.

The validity of correcting the Co-Ral absorbance at 290 mμ for that of chloromethylumbelliferone by measuring the latter at 380 mμ after adding sodium carbonate depends on the fact that Beer's law is obeyed in each of the three cases. This was tested and the results showed that there are no deviations from Beer's law in the concentration ranges required.

The stability of solutions of Co-Ral and chloromethylumbelliferone in methanol and of the latter compound in aqueous methanolic sodium carbonate were determined. Co-Ral and chloromethylumbelliferone did not change in absorbance at wave length 290 mμ during an 8-hour period. In 48 hours the Co-Ral solution decreased in absorbance by about 5%, while the chloromethylumbelliferone decreased by about 18%. The solution of chloromethylumbelliferone in sodium carbonate did not change appreciably in 15 minutes. Therefore, care must be taken to read the absorbance of these solutions immediately after adding the sodium carbonate. In 1.5 hours the sodium carbonate solution of chloromethylumbelliferone decreased in absorbance by about 8%.

In the determination of the Co-Ral content of technical material, dilute hydrochloric acid must be added before determining the absorbance in absolute methanol at 290 mμ. This is necessary because of the presence of some potassium carbonate in the technical material. The hydrochloric acid has no effect on the absorbance of Co-Ral.

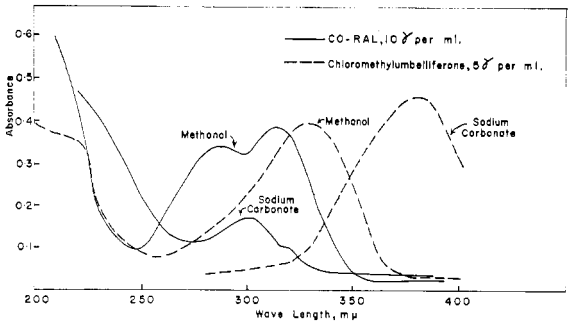


Figure 3. Ultraviolet absorption spectra of Co-Ral and chloromethylumbelliferone in absolute methanol and in 50% methanol containing sodium carbonate

Table I. Absorbance of Co-Ral and Chloromethylumbelliferone

Compound	Solvent	Wave Length, Mμ	Absorbance, $E^{1\%}_{1cm.}$
Co-Ral	Methanol	290	297
Chloromethylumbelliferone	Methanol	290	200
	Aqueous methanolic sodium carbonate	380	912

Analytical Procedures

Hydrolysis Method. REAGENTS. Only reagent grade chemicals should be used.

POTASSIUM HYDROXIDE, 1*N* solution in absolute isopropyl alcohol. Dissolve 66 grams of potassium hydroxide in 1 liter of isopropyl alcohol. Allow the insoluble material to settle. Use the supernatant liquid.

HYDROCHLORIC ACID, 0.5*N*, accurately standardized.

Procedure. Weigh about 1 gram of the Co-Ral sample to be analyzed into a 250-ml. Erlenmeyer flask. Add 25 ml. of 1*N* potassium hydroxide and reflux for 2 hours under a reflux condenser equipped with a soda-lime tube. Wash down the condenser with distilled water and transfer the contents of the flask to 250-ml. beaker. Adjust the final volume of solution to about 100 ml. with distilled water. Insert a calomel-glass electrode system and titrate to pH 7.5 with 0.5*N* hydrochloric acid. Carry out a blank determination. The titer difference between the sample and blank is converted to Co-Ral equivalents. One milliliter of 0.5*N* hydrochloric acid is equivalent to 60.47 mg. of Co-Ral.

The method can be applied to a 25% wettable powder by weighing a 4-gram sample into a Whatman extraction thimble and extracting with acetone for 1 hour in a Bailey-Walker extractor. After evaporation of the acetone, the procedure is employed as above.

Spectrophotometric Method. REAGENTS. Only reagent grade chemicals should be used.

Co-Ral, Standard Solution. Dissolve exactly 0.200 gram of Co-Ral (recrystallized, melting point 94° C.) in absolute methanol. Warm if necessary to dissolve. Dilute to 100 ml. with absolute methanol. This solution contains 2000 γ per ml. of Co-Ral. Make a further 1 to 20 dilution of the stock solution with absolute methanol. From this second solution, prepare fresh daily standards by diluting 10-, 15-, and 20-

ml. aliquots to 100 ml. with absolute methanol. The resulting solutions will contain, respectively, 10, 15, and 20 γ per ml. of Co-Ral.

Procedure. Weigh accurately approximately 0.2 gram of technical Co-Ral and place in a 250-ml. volumetric flask. Add 100 ml. of 1,4-dioxane and 5 ml. of 0.1N hydrochloric acid, stopper, and shake until the Co-Ral is dissolved. Dilute to volume with absolute methanol (Solution 1). Pipet a 2-ml. aliquot of the solution into a 100-ml. volumetric flask, and again dilute to volume with absolute methanol (Solution 2). Measure the absorbance in a 1-cm. silica cell at 290 mμ in a suitable spectrophotometer. Use a 0.8% v./v. solution of 1,4-dioxane in absolute methanol as a blank.

For the estimation of chloromethylumbelliferone, pipet 50 ml. of Solution 1 into a 100-ml. volumetric flask. Pipet in 50 ml. of 1% aqueous sodium carbonate solution. Cool rapidly to room temperature and dilute to volume with absolute methanol. Within 15 minutes of the sodium carbonate addition, read the absorbance in a 1-cm. cell at 380 mμ against a blank solution containing the same proportions of methanol, 1,4-dioxane, and aqueous sodium carbonate as are present in the sample solution. Calculate the chloromethylumbelliferone content by reference to the appropriate calibration curve. Multiply the chloromethylumbelliferone concentration by 0.67 and subtract from the Co-Ral value obtained at wave length 290 mμ.

Discussion

Reproducibility. The reproducibility of both methods is shown in Table II. The results show that the hydrolysis procedure is capable of somewhat greater precision than the spectrophotometric method. This is to be expected. However, the reproducibility of the spectrophotometric procedure is satisfactory for analytical control purposes. The precision of the spectrophometric method is reduced by running duplicate determinations. In general, the spectrophotometric method is preferred, because it permits a ready correction for chloromethylumbelliferone.

Table II. Reproducibility of Methods for Co-Ral Determination

Method	Material	Mean Value[a]	Standard Deviation
Hydrolysis	Recrystallized	100.73 (9)	0.15
Spectrophotometric	Technical	96.3 (4)	0.6
		94.9 (4)	0.5
		93.8 (4)	0.5
		92.3 (4)	0.6

[a] Number of values used to calculate each mean shown in parentheses.

The accuracy of the method for determination of chloromethylumbelliferone in Co-Ral was tested by preparing synthetic mixtures of the two compounds. The chloromethylumbelliferone results are shown in Table III. It is apparent that the method described will give very accurate values for chloromethylumbelliferone in the presence of Co-Ral.

Table III. Determination of Chloromethylumbelliferone Content of Co-Ral

Chloromethylumbelliferone, %	
Present	Found
0.99	0.99
0.99	0.99
2.00	1.98
2.01	2.00
3.02	3.01
3.01	2.99

The presence of more than small amounts of chloromethylumbelliferone can be detected by the hydrolysis method, although the actual concentration of interfering material cannot be determined accurately by this method. The presence of chloromethylumbelliferone will result in a change in the relative number of moles of acid obtained on titration to pH 7.5 and 3.0. In the case of pure Co-Ral, this ratio is 3 to 2 (Figure 1), while for chloromethylumbelliferone it is 1 to 1.

Acknowledgment

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FUNGICIDE ASSAY

Determination of Dyrene and Cyanuric Chloride in Technical Materials

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Dyrene (2,4-dichloro-6-*o*-chloroanilino-*s*-triazine), previously known as Kemate or B-622, is a new organic fungicide which has proved outstandingly effective for the control of fungus diseases on a number of crops. It is prepared by the reaction of cyanuric chloride with *o*-chloroaniline in the presence of sodium carbonate. This study was undertaken to develop a suitable assay method for this product in the presence of possible contaminants.

A residue method for Dyrene (*5*) is based on acid hydrolysis to form *o*-chloroaniline. The *o*-chloroaniline is then diazotized and coupled with *N*-1-naphthylethylenediamine to produce a colored complex. This method is time-consuming, as the hydrolysis requires 2 hours of reflux with hydrochloric acid. A second colorimetric procedure involving the Zincke reaction (*2*) has also been described. In general, volumetric methods are more accurate than colorimetric procedures in cases where the former can be employed. Therefore, it was decided to investigate the hydrolysis of the chlorine atoms of Dyrene, because chloride ion can be readily determined volumetrically. A second volumetric