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Effects of Organochlorine Insecticides on Metabolism of Cholecalciferol (Vitamin D_3) in Rachitic Cockerel

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The metabolic pathway involved in the conversion of cholecalciferol (CC) to its biologically active form is: CC $\xrightarrow{\text{Liver}}$ 25-OH-CC $\xrightarrow{\text{Kidney}}$ 1,25-diOH-CC. The most predominant form of the steroid in the target intestine is 1,25-diOH-CC. It is known that CC must undergo at least these two conversions prior to stimulating intestinal calcium transport. Using the blood levels of 25-OH-CC as an indication of liver function, it was found that organochlorine pesticide treatment did not influence this hydroxylation step, whereas the amount of 1,25-diOH-CC in intestine of chicks exposed to pesticide was slightly more than in untreated chicks. 1,25-diOH-CC was shown to be homogenous and the same in control and organochlorine insecticide-treated intestines by both sensitive silicic acid and Celite column chromatography. It is concluded from this data that cholecalciferol is converted to its biologically active forms in sufficient quantity in the presence of organochlorine insecticides to maintain normal calcium metabolism in the chick. These results do not then explain how organochlorine insecticides impair the biological responses to cholecalciferol.

Pro- and anti-DDT scientific papers have proliferated since Rachel Carson's book *Silent Spring* (1962), *e.g.*, some 50,000 papers have appeared. The insecticide DDT commonly used is technical grade with a composition of 79% of *p,p'*-DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane] and 20% *o,p'*-DDT [1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane]. Perhaps the most widely agreed upon ecological hazard of DDT is the decline in the population of several raptorial birds (Ratcliffe, 1967; Porter and Wiemeyer, 1969; French and Jefferies, 1969; Hickey, 1969; Bengelsdorf, 1969; Edwards, 1970; Robinson, 1970; Moser, 1971). These failures in reproduction have been shown to result from a lesion in calcium metabolism, and thus thinning of the eggshell. As presented in the previous paper, a possible partial explanation for the thin eggshell syndrome may be that DDT interferes in the known biological activities of cholecalciferol (vitamin D_3), a major steriod regulator of calcium metabolism.

The concept has been developed in a number of laboratories (Haussler *et al.*, 1968; Lawson *et al.*, 1969; Myrtle *et al.*, 1970; Cousins *et al.*, 1970) that cholecalciferol (CC) must undergo at least two metabolic conversions prior to stimulating intestinal calcium transport. It has been conclusively

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shown that administration of radioactive cholecalciferol to rachitic chicks or rats results in the production of two major polar metabolites and a number of more minor metabolites. The predominant metabolite present in the blood has been shown by Blunt *et al.* (1968a,b) to be 25-hydroxycholecalciferol (25-OH-CC). Horsting and DeLuca (1969) have shown this hydroxylation reaction to be located in the liver.

The major metabolite of cholecalciferol present in the target intestine was shown by Norman and coworkers to be chemically different from both the parent vitamin and 25-OH-CC. The highly selective nature of the binding of the chemically different form to the target intestine and the kinetics of appearance of this metabolite in relation to the well known lag in action of vitamin D (Haussler *et al.*, 1968; Myrtle *et al.*, 1970) was suggestive that it played a prominent role in the development of intestinal calcium transport.

Lower concentrations of this metabolite were also found in bone and kidney of chicks and in the eggshell gland of the laying hen, other known sites of intensive calcium metabolism, and in the intestinal mucosa of the frog, rat, rabbit, monkey, and man (Norman *et al.*, 1971a). Myrtle and Norman (1971a) reported that the metabolite was four to five times as effective as cholecalciferol and over two times as effective as 25-OH-cholecalciferol in stimulating intestinal calcium transport 24 hr after administration. Following a considerable lag, cholecalciferol and its 25-hydroxy derivative produce a maximum stimulation of the transport response at 24 to 48 hr. Most significantly this metabolite greatly shortened this lag, stimulating maximum calcium transport by 9 hr. At 9 hr this metabolite is at least 13 times as active as the parent vitamin. Similar results were also obtained by Haussler *et al.* (1971).

This metabolite has been identified independently in three different laboratories by Lawson *et al.* (1971), Norman *et al.* (1971c), and Holick *et al.* (1971) to be 1,25-dihydroxycholecalciferol (1,25-diOH-CC). The intermediate, 25-OH-CC, is converted to the more polar metabolite 1,25-diOH-CC by the kidney (Norman *et al.*, 1971b; Fraser and Kodicek, 1970; Gray *et al.*, 1971).

Accordingly we have chosen to explore the possible interaction between the nutritional stress of CC deficiency and the toxicological stress of exposure to organochlorine insecticide. Inasmuch as CC is a principle regulator of calcium metabolism, it was of interest to determine the effect of organochlorine insecticide on the pathway and level of CC metabolites in various body pools. The primary purpose of this paper is to assess if organochlorine insecticides alter the pathway or levels of biologically active metabolite(s) of cholecalciferol in the chick.

MATERIALS AND METHODS

Materials. 4-^{14}*C*-Cholecalciferol (Philips-Duphar, Amsterdam), sp act. 20.8 mCi/mmol, and 1,2-^{3}*H*-cholecalciferol (New England Nuclear), sp act. 38.8 mCi/mmol, were utilized in the metabolism studies.

Silicic acid (minus 325 mesh) was obtained from Calbiochem. Celite was obtained from Johns-Manville. All solvents and chemicals used were analytical grade.

Chickens. One-day-old White Leghorn cockerels, generously provided by H&N of California, Inc., were employed in all experiments. They were housed in electrically heated tier brooders in an air-conditioned room from which sunlight was excluded. The chicks were maintained on a vitamin D-deficient diet whose composition has been previously described by Hibberd and Norman (1969). After 3 weeks on this diet their growth rate leveled off, and they were clearly rachitic. Chicks were given either *p,p'*-DDT or *o,p'*-DDT orally, dissolved in 1,2-propanediol when they were either 1- or 2-weeks-old in the majority of experiments. In experiments designed to expose the chicks for a longer period of time to the chlorinated hydrocarbon, chicks were dosed when they were 3-days-old. This is necessary because rachitic chicks begin to die in the fourth week at a high rate. Chicks were dosed at 1 or 5 mg/day in a volume of 0.20 ml; controls were given equal amounts of the vehicle. Standard cholecalciferol doses were prepared by dissolving the weighed steroid (Philips-Duphar, Weesp, The Netherlands) in a minimum of diethyl ether. 1 IU (International Unit) of cholecalciferol (vitamin D_3) is equivalent to 0.025 μg (65 pmol). Hibberd and Norman (1969) determined the minimum daily requirement for cholecalciferol in the chick to be 0.65 to 1.30 nmol (10 to 20 IU). The appropriate volume of 1,2-propanediol was added, the ether was removed by warming and bubbling through nitrogen, and the ultraviolet spectrum and absolute concentration were determined by using a Beckman DB recording spectrophotometer.

At various stages of insecticide treatment, lasting from 1 to 3 weeks, five chicks from each group, treated and untreated, were administered 10 IU of either ^{14}C-CC or ^{3}H-CC, respectively, and the metabolism assayed either 3 or 15-16 hr later as described below (double label methods).

Lipid Extraction Techniques. Lipid extraction of the liver and small intestine was carried out according to a modification of the procedure of Bligh and Dyer (1959), as described by Haussler *et al.* (1968). Small intestines (duodenum, illeum, and jejunum) were removed and the contents squeezed out. The intestines were then slit lengthwise and rinsed with ice cold 0.90% NaCl. The total lipid was extracted by blending the tissue, after cutting into small segments, in methanol-chloroform (2:1) in a Waring Blendor. Heparinized whole blood was mixed with five parts of methanol-chloroform (2:1). Protein was subsequently removed by centrifuging. The protein pellet was reextracted with methanol-chloroform and the protein again removed by centrifugation. The two organic extracts were combined. Chloroform and water were added to the combined extracts to cause a phase separation of aqueous methanol and chloroform. The chloroform layer was removed and stored under nitrogen at $-20°C$.

Chromatography. The chloroform lipid extract of interest was brought to dryness with a stream of nitrogen, redissolved in petroleum ether, and dried over anhydrous Na_2SO_4. The petroleum ether was transferred to a second container, an aliquot was taken, and the remainder was brought to a small volume with a stream of nitrogen. Next, 0.5 ml of water was added to the lipid sample, and the sample was applied to a 30-g (1 $\times$ 80 cm) silicic acid column. Elution of the various classes of lipids was accomplished with a 250-ml constant volume mixing chamber to generate successive exponential gradients of solvents with increasing dielectric constant. The mixing chamber was first filled with 250 ml of petroleum ether. The elution system was similar to that described by Haussler *et al.* (1968), except that the holding chamber was filled successively with 300 ml of 100% diethyl ether, 500 ml of 50% (v/v) 1,2-dichloroethane in diethyl ether, 300 ml of 100% acetone, and 300 ml of 100% methanol. Fractions of 10 ml were collected. This chromatography system resolves the tissue radioactivity into three major radioactive compounds: unmetabolized cholecalciferol, 25-hydroxycholecalciferol, and the more polar compound, 1,25-diOH-CC. Several minor polar metabolites are also resolved in this system. Celite

Table I. Effects of Short-Term Organochlorine Insecticide Treatment on the 16-Hr Metabolism of 10 IU (0.65 nmol) of Cholecalciferol

	pmol of metabolite/100 g chick		
Intestine[a]	CC	25-OH-CC	1,25-diOH-CC
-CC, *o,p'*-DDT	1.13	2.04	4.93
-CC, *p,p'*-DDT	0.57	1.89	5.47
-CC (1,2-propanediol)	0.91	2.15	3.01
-CC (untreated)	0.47	1.75	3.24
	pmol of metabolite/ml of plasma		
Plasma	CC	25-OH-CC	Peak 4B
-CC, *o,p'*-DDT	2.57	63.2	7.03
-CC, *p,p'*-DDT	2.30	59.2	6.77
-CC (1,2-propanediol)	3.74	61.9	6.90
-CC (untreated)	2.45	64.8	7.62

[a] Intestine and plasma results were corrected to a standard animal weight and plasma volume, respectively, to negate differences due to minor variation in animal size. The amounts of metabolites were determined by assuming the same specific activity as the parent cholecalciferol with the exception of intestinal 1,25-diOH-CC, which was corrected for a 17% tritium loss.

liquid-liquid partition chromatography was performed as previously described by Engel *et al.* (1961). Fractions of 10 ml were collected. The Celite partition chromatography system is capable of separating steroids as closely related as estriol and 16-epiestriol.

Radioactivity Determinations. Liquid scintillation counting of tritium and ^{14}C-labeled steroids was carried out in a Beckman model CPM 200 counter equipped with external standardization. All samples were dissolved in 10 ml of a counting solution consisting of 5 g of phenylbiphenyloxadiazole-1,3,4 (PBD) per liter of toluene and counted to 2% error. The number of disintegrations per min of tritium and carbon-14 present in the sample was determined by the use of external standardization and a computer program designed to process double label counting data. With the use of the specific activities of the parent cholecalciferol and the number of chicks used in the experiment, this program also converted the disintegrations per min in the sample to absolute amount (pmoles) of steroid per sample per chick. The amounts of metabolite were determined by assuming the same specific activity as the parent cholecalciferol with the exception of intestinal 1,25-diOH-CC, which was corrected for a tritium loss of 17% (Myrtle and Norman, 1971b).

RESULTS AND DISCUSSION

Organochlorine insecticides such as *p,p'*-DDT and *o,p'*-DDT have been implicated in the alteration of calcium metabolism in adult birds. In addition, such chemicals have been shown to induce hydroxylase enzymes in tissues such as the liver, leading to the hydroxylation and premature excretion of steroids. To test whether *p,p'*-DDT or *o,p'*-DDT altered calcium metabolism through an effect on the metabolic conversion of cholecalciferol to its biologically active metabolites, cholecalciferol metabolism was studied in rachitic chicks previously treated with *p,p'*-DDT or *o,p'*-DDT and untreated rachitic chicks.

The data contained in Table I convincingly established that the metabolic pathway of cholecalciferol metabolism was not significantly altered due to pretreatment with *o,p'*-DDT or *p,p'*-DDT. As can be seen, the amount of the steroid converted to 25-OH-CC, the major circulating form, was not significantly altered. Likewise the conversion of 25-OH-CC to 1,25-diOH-CC, the proposed active form of cholecalciferol, was not decreased by pesticide pretreatment. This experimental design had a short interval between exposure to the chlorinated hydrocarbon and the study of cholecalciferol metabolism. This dosing regimen results in a deleterious intervention in the known biological activities of cholecalciferol (Nowicki *et al.*, 1971).

Table II contains the results of a similar study which was designed to expose the chicks to a higher dose of the insecticide for a longer period of time. The birds which are having reproductive failures, apparently due to abnormal calcium metabolism, are those that are feeding on trophic levels which accumulate chlorinated hydrocarbons for long periods before development of the eggshell-thinning phenomenon. Thus,

Table II. Effect of Long-Term Administration of *o,p'*-DDT on the 16-Hr Metabolism of 10 IU (0.65 nmol) of Cholecalciferol[a]

	1-Week pretreatment		
	pmol of metabolite(s)/chick		
	CC	25-OH-CC	1,25-diOH-CC
Liver			
-CC, *o,p'*-DDT	0.67	3.11	0.97
-CC, control	0.69	2.92	0.71
Blood			
-CC, *o,p'*-DDT	0.80	74.12	6.40
-CC, control	1.28	72.56	6.42
Intestine			
-CC, *o,p'*-DDT	0.41	2.45	8.75
-CC, control	0.47	2.12	6.94
	2-Weeks pretreatment		
	pmol of metabolite(s)/chick		
	CC	25-OH-CC	1,25-diOH-CC
Liver			
-CC, *o,p'*-DDT	0.83	2.59	0.96
-CC, control	0.81	2.69	1.27
Blood			
-CC, *o,p'*-DDT	0.56	66.64	4.32
-CC, control	0.88	68.16	4.61
Intestine			
-CC, *o,p'*-DDT	0.69	2.24	9.56
-CC, control	0.73	2.76	7.16
	3-Weeks pretreatment		
	pmol of metabolite(s)/chick		
	CC	25-OH-CC	1,25-diOH-CC
Liver			
-CC, *o,p*-DDT	0.79	2.79	0.98
-CC, control	0.73	2.83	0.78
Blood			
-CC, *o,p'*-DDT	2.88	68.08	4.92
-CC, control	2.45	67.84	4.48
Intestine			
-CC, *o,p'*-DDT	0.79	2.35	9.07
-CC, control	0.83	2.26	7.00

[a] Twenty-week-old rachitic chicks received 5 mg of *o,p'*-DDT per day dissolved in 0.20 ml of 1,2-propanediol and another twenty were given 0.20 ml of vehicle (control). After 1 week five chicks from each group were dosed with 10 IU (0.65 nmol) of cholecalciferol; *o,p'*-DDT pretreated received 4-^{14}C-CC, and controls received 1,2-^{3}H-CC. The chicks were sacrificed 16 hr later and the metabolism assayed as described in Methods. The remaining chicks were dosed with 5 mg of *o,p'*-DDT daily. At the end of 2 and 3 weeks, another five chicks from each group were removed and dosed with 10 IU of CC. The 1,25-diOH-CC region of the blood and liver chromatogram is known to be heterogeneous.

this type of an experiment approaches the situation of the bird in the field. As can be seen, the results (Table II) were similar to those found in Table I, *e.g.*, these levels of chlorinated hydrocarbon did not alter the pathway or deleteriously affect the level of active form(s) produced from the parent cholecalciferol.

Although calciferol is the major steroid regulator of calcium metabolism, our data has convincingly established that the effect of organochlorine insecticides in causing thin eggshells and thus reproductive failure in several predatory birds cannot be ascribed simply to changes in the metabolism of cholecalciferol to its biologically active forms, 25–OH–CC and 1,25-diOH–CC. The amount of CC converted to 25–OH–CC, the major circulating form, and 1,25–diOH–CC, the major form in the intestine, was not influenced deleteriously by pretreatment with organochlorine insecticide.

When the metabolism of cholecalciferol is studied 15–16 hr after dosing, essentially all of the steroid is metabolized to polar forms, at least two of which are more biologically active than the parent. This time interval of metabolism, 15–16 hr, provided information on amounts of polar forms in various body pools which are essential for normal calcium metabolism. These results established that CC is converted to its metabolites in sufficient quantity in order to maintain normal calcium metabolism. However, this type of an experiment (15–16 hr metabolism) does not give any information concerning the early time interval of metabolism. Obviously, in order to demonstrate an induction of metabolism of CC, it is necessary to study the metabolism at a time when there is some unaltered substrate available. Accordingly, metabolite(s) distribution was studied 3 hr after administration of labeled CC (Table III). As can be seen from Table III, pretreatment with *p,p'*-DDT did not deleteriously alter the rate of conversion of CC to its two major biologically active metabolites in blood, 25–OH–CC, and intestine, 1,25–diOH–CC.

As compiled in Table IV, pretreatment with *o,p'*-DDT or *p,p'*-DDT did not induce the hepatic conversion of CC to 25–OH–CC, using the circulating level of 25–OH–CC as an index of liver function. However, the kidney conversion of the intermediate, 25–OH–CC, to 1,25–diOH–CC was apparently induced, using the amount of 1,25–diOH–CC in the intestine as an index of kidney function. These data suggest that the conversion of 25–OH–CC to 1,25–diOH–CC by the kidney has undergone an induction or activation of the factors concerned in the biosynthetic reaction.

Table III. Effects of *p,p'*-DDT on the 3-Hr Metabolism of 10 IU (0.65 nmol) of Cholecalciferol[a]

	pmol of metabolite(s)/chick		
	CC	25–OH–CC	1,25–diOH–CC
Liver			
–CC, *p,p'*-DDT	30.1	170.00	29.7
–CC, control	34.1	168.00	20.6
Blood			
–CC, *p,p'*-DDT	79.8	119.00	5.8
–CC, control	80.16	122.00	5.8
Intestine			
–CC, *p,p'*-DDT	5.95	2.66	0.73
–CC, control	5.89	2.74	0.51

[a] Two-week-old rachitic chicks received 5 mg of *p,p'*-DDT per day for 7 days, at which time five were given 10 IU (0.65 nmol) of 4–^{14}C-CC. Five untreated chicks were also given 10 IU of 1,2–^{3}H-CC; both groups were sacrificed 3 hr after dosing and analyzed as described in the Methods. The 1,25–diOH–CC region of the blood and liver chromatogram is known to be heterogeneous.

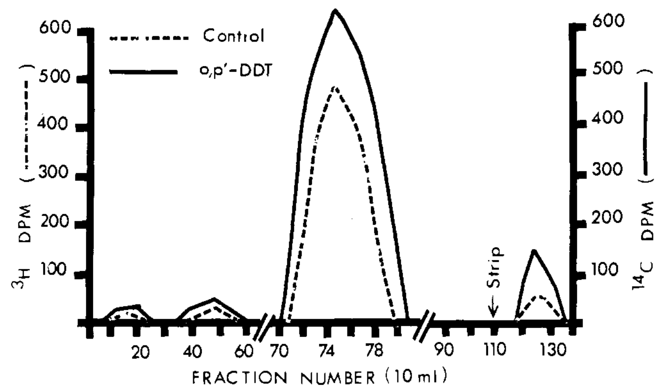

Figure 1. Celite column chromatography of 1,25-dihydroxycholecalciferol (1,25–diOH–CC) from control (nonpesticide treated) and *o,p'*-DDT treated intestine. The double-labeled 1,25–diOH–CC used for Celite column chromatography was isolated from the fractions known to contain 1,25–diOH–CC off of a silicic acid column, as described in the Methods

Table IV. Relative Comparison of the Amount of 25-Hydroxycholecalciferol in Blood and 1,25-Dihydroxycholecalciferol in Intestine in Organochlorine Treated and Untreated Rachitic Cockerels

Source of data	25–OH–CC organochlorine treated (pmol/chick) / 25–OH–CC organochlorine untreated (pmol/chick) Blood	1,25–diOH–CC organochlorine treated (pmol/chick) / 1,25–diOH–CC organochlorine untreated (pmol/chick) Intestine
Table I, *o,p'*-DDT	1.02	1.64
Table I, *p,p'*-DDT	0.96	1.82
Table II, *o,p'*-DDT (1-week)	1.01	1.26
Table II, *o,p'*-DDT (2-week)	0.98	1.34
Table II, *o,p'*-DDT (3-week)	1.00	1.30
Table III, *p,p'*-DDT	0.98	1.43

Since 1,25–diOH–CC isolated from both organochlorine treated and untreated chick intestines migrated together during silicic acid column chromatography, it was of interest to determine homogeneity in a more sensitive chromatography system. Thus, Celite liquid–liquid partition column chromatography, which can separate the 16-epiestriols, was employed.

The 1,25–diOH–CC regions of the silicic acid column chromatogram from insecticide-treated and nontreated chicks were combined and rechromatographed on the Celite column. The results contained in Figure 1 indicate that the major CC metabolite present in the intestine of pesticide-treated chicks migrates identically with the 1,25–diOH–CC present in the intestine of control chicks. 85% of the tritium in the control intestine migrated as a symmetrical peak with the mobility predicted for 1,25–diOH–CC. More importantly, 89% of the carbon-14 from the *o,p'*-DDT pretreated chick small intestine migrated identically with the 1,25–diOH–CC of the control intestine. Thus, the most biologically active form of cholecalciferol, 1,25–diOH–CC, in stimulating intestinal calcium transport, present in both pesticide- and nonpesticide-treated chicks, was chemically identical, *e.g.*, there was no subtle chemical modification of 1,25–diOH–CC in pesticide treated chicks.

Clearly, a great deal of work yet remains in order to clarify the biochemical lesion caused by *o,p'*-DDT or *p,p'*-DDT which results in the blockage of the known biological functions of cholecalciferol, since these compounds do not have a deleterious effect on the metabolism of the vitamin to its more active forms. Also, the extrapolation of our data to the bird in the field is difficult. We are presently carrying out further experiments in an attempt to define the relationship of the known biological activities of vitamin D to the thin eggshell syndrome.

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Metabolism of Cyclophosphamide by Sheep

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Eight sheep urinary metabolites from a single oral dose of cyclophosphamide-^{14}C (I) have been either identified or characterized. These metabolites represented approximately 95% of the urinary radioactivity from ring-^{14}C-labeled I and 88% of the urinary radioactivity from side chain-^{14}C-labeled I. The major route of excretion was in the urine (70–90% of the dose) and the major route of metabolism was oxidation of the ring followed by hydrolysis.

Cyclophosphamide (I, Figure 1) is a nitrogen mustard derivative which has been used in the treatment of many types of cancer (Nissen-Meyer and Host, 1960; Sullivan, 1967; Haggard, 1967; Sutow, 1967). It also causes a temporary loss of hair. Homan *et al.* (1968) and Dolnick *et al.* (1969) have reported the application of this latter property to the defleecing of sheep.

The purpose of the present study was to determine the chemistry of the residues that would result from the use of cyclophosphamide as a chemical defleecing agent.

We have previously reported (Bakke *et al.*, 1971) the identification of two of the major urinary metabolites (Compounds II and VI, Figure 1). These resulted from oxidation of I at the 4 position of the tetrahydro-2*H*-1,3,2-oxazaphosphorine ring to yield 4-ketocyclophosphamide (II), and hydrolysis of II to yield the phosphorodiamidate (VI). These have been identified by Hill *et al.* (1970) and Struck (1971) in the urine from dogs given cyclophosphamide by intravenous injection.

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EXPERIMENTAL

Purification of Cyclophosphamide. A solution of 0.242 g (1.5 μCi) of side chain-labeled cyclophosphamide (New England Nuclear Corp., Boston, Mass.) and 3.758 g of unlabeled cyclophosphamide monohydrate in 6 ml of acetone was placed on a 2.9 × 17 cm column of acid-washed alumina (Merck 71695) packed in acetone. The column was eluted slowly with acetone–ethanol (9:1), and the eluents were monitored with a radioactive flow monitor equipped with a cerium-activated glass bead cell. The large peak that eluted was collected, except for its leading and trailing edges, and the solvents were evaporated with a flash evaporator at water aspirator vacuum. A small amount of 2-methyl-4-keto-2-pentanol (acetone dimer) which