# Mechanism of Formation of Chloropropanols Present in Protein Hydrolysates

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**Chloropropanols are formed in protein hydrolysates by the reaction of hydrochloric acid with residual lipids associated with the proteinaceous materials used in their production. The products formed from glycerol, triolein, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine and soya meal have been analyzed by thin-layer and gas chromatography. The yields and isomer ratios of the chloropropandiols and dichloropropanols formed are interpreted in terms of reaction mechanisms for their formation, which involve preferential nucleophilic substitution by the chloride anion at positions activated by neighboring ester groups. These provide anchimeric assistance and govern regioselectivity through steric and electronic effects.**

KEY WORDS: Chloropropanols, glycerides, glycerol, hydrochloric acid, nucleophilic substitution, phospholipids, protein hydrolysates.

Protein hydrolysates are widely used as seasonings and ingredients in processed savory food products. They are commonly produced by hydrochloric acid hydrolysis of proteinaceous by-products from edible oil extraction, such as soybean meal, rapeseed meal and maize gluten. Studies by Velisek *et al.* (1–6) have demonstrated the presence in protein hydrolysates of several chloropropanols and their fatty acid esters, and their formation in model hydrolysis systems from lipids (both synthetic and residual lipids from raw materials used in hydrolysate manufacture). The main chloropropanol detected by Velisek in protein hydrolysates was 1,3-dichloropropan-2-ol (1,3DCP), together with smaller amounts of 2,3-dichloropropan-1-ol (2,3DCP) and 3-chloropropanol (4,6). Model studies with lipids (3) and glycerol (2) strongly suggest that 3-chloropropan-1,2-diol (3MCPD) and 2-chloropropan-1,3-diol (2MCPD) may also be expected in commercial hydrolysates, but to date no methods for their determination or data on their levels in protein hydrolysates have been published.

In a recent publication (7) we report methods of analysis suitable for the confirmed determination of a range of chloropropanols in protein hydrolysates and derived food products. The methods permit the quantitative determination of 3MCPD, 2MCPD, 1,3DCP and 2,3DCP, the principal chloropropanols found in traditionally processed hydrolysates.

Using these analytical methods we have investigated the mechanisms by which these chloropropanols are formed from glycerol, its esters and soybean meal.

## MATERIALS AND METHODS

*Materials.* Anhydrous sodium sulphate and diethyl ether were analytical reagent (AR) grade from Fisons (Poole, U.K.), while sodium hydroxide and sodium bicarbonate (AR grade) were obtained from BDH (Poole, U.K.). Extrelut 20 columns were obtained from Merck (Darmstadt, Germany). Heptafluorobutyryl imidazole (HFBI) was obtained from Pierce (Oud-Beijerland, The Netherlands), hexane from Rathburn Chemicals Ltd. (Walkerburn, Scotland), 3-chloropropanol-1,2-diol (calibration standard) from Fluka (Buchs, Switzerland), 1,3-dichloropropan-2-ol (1,3DCP) and 2,3-dichloropropan-1-ol (2,3DCP) from Pfaltz & Bauer (Stamford, CT), p-dichlorobenzene (PDCB) from Aldrich (Eillingham, U.K.), glycerol-d5 (penta-deuterated) from Merck Sharpe & Dohm Isotopes, and oleic acid and triolein from Sigma Chemical Co. (St. Louis, MO). 1,2-Diacylphosphorylcholine (PC) was purified from soya lecithin and 2-chloropropan-1,3-diol was provided by Dr. F. Ruf (Maizena GmBh, Heilbron, Germany).

*Hydrolysis reactions.* Unless stated differently elsewhere, reaction conditions were as follows: Substrate ($1 \times 10^{-4}$ moles) was weighed into a 2-mL septum-capped glass vial. Hydrochloric acid (1 mL, 5.5 M) was added and the vial was capped, shaken and heated at 107°C for 16 hr.

*Sample preparation.* The reaction vial containing the reaction mixture was cooled to ambient temperature and uncapped, and its contents were quantitatively transferred, by using distilled water, to a graduated test tube (25 mL). Each sample was adjusted to 20 mL and neutralized by the addition of distilled water and sodium hydroxide solution (40% w/v). The neutralized solution was applied to an Extrelut 20 column (Merck) and allowed to equilibrate for 15 min (Note: viscous samples may be mixed with Extelut refill material before it is packed into a column). Chloropropanols were recovered from Extrelut columns as follows: For analysis of all chloropropanols (mono-ols and diols), a two-stage elution separating the less abundant mono-ols from the more abundant diols and other interfering compounds proved necessary. The Extrelut 20 column was first eluted with hexane/diethyl ether (90:10, v/v) to collect 50 mL (eluate 1) containing the mono-ols, followed by diethyl ether to collect 250 mL (eluate 2) containing the chloropropandiols. For analysis only of the chloropropandiols the Extrelut column was eluted simply with diethyl ether to collect 250 mL (eluate 3).

*Derivatization.* Eluate 1 (5 mL), or eluate 2 or 3 (1 mL), or the calibration solution (1 mL) was pipetted into a 25-mL volumetric flask. PDCB standard solution (1 mL) was made up to 25 mL with hexane. HFBI (200 μL) was added, the solution was mixed and allowed to stand at ambient temperature (20°C) for 15 min with intermittent shaking.

The mixture was then transferred to a screw-capped vial containing distilled water (2 mL) and shaken (1 min), the separated organic layer was washed twice more, and a 10-mL aliquot was filtered through a 4-cm column of anhydrous sodium sulphate (4 g) before analysis by gas chromatography (GC). Dried derivatized solutions were found to be stable for up to three days under refrigeration. When necessary, limits of determination were improved by taking a larger aliquot of the appropriate Extrelut fraction for derivatization. When aliquots greater than 2 mL are taken, ether eluates 2 and 3 must be blown dry under a gentle stream of nitrogen before derivatization to overcome sporadic ether-based interference problems.

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Individual chloropropanols were quantitated by analysis with a capillary gas chromatograph and an electron capture detector (GC-ECD), while total chloropropandiols were quantitated by packed-column gas chromatography and an ECD.

*Analysis by GC-ECD.* Heptafluorobutyrate esters of the chloropropanols were separated and determined by gas chromatography with electron capture detection, using a Perkin Elmer 8320 (Perkin Elmer, Norwalk, CT) or a Philips PU 4550 gas chromatograph (Philips, Cincinnati, OH) fitted with a splitless injection port. Operating conditions were optimized as follows: Column: 25 m × 0.2 mm fused silica with 0.33-μm film of immobilized OV 1 (part No. 19091Z-1020, Hewlett Packard, Palo Alto, CA). Oven temperature program: 50°C for 2 min, 50°C to 85°C at 1°C/min, 85–250°C at 20°/min, hold at 250°C for 20 min. Helium carrier gas: 12 psig. Injector: Splitless at 280°C, vent opened after 0.6 min. Injection volume: Up to 3 μL. EC detector temperature: 350°C (Perkin Elmer 8320). Detector responses to derivatized chloropropanols and the PDCB internal standard vary with temperature in an instrument-dependent fashion. The ECD should be operated within a temperature range over which detector response is least affected by temperature change.

*Analysis by packed column GC.* A Pye Unicam (Cambridge, U.K.) 304 gas chromatograph fitted with an ECD and operating with an injection temperature of 140°C and oven temperature of 125°C (isothermal) was used. Separation was carried out on 10% SP-2250 supported on 100/120 Supelcoport packed in a 5 ft × 0.4 cm column; nitrogen was used as the carrier gas at 30 mL/min.

The combined chloropropandiol peak had a 1.9-min retention time and was quantitated by comparison of the peak height with that of a calibration graph. The calibration graph was constructed from the peak heights of a range of standard solutions of 3MCPD prepared in ether and derivatized alongside the sample solutions.

*Thin-layer chromatography.* Thin-layer chromatographic analysis was carried out on precoated silica plates; solvent mixtures used for development were chloroform/methanol/acetic acid/water (85:15:10:3, v/v) and petroleum ether/ether/formic acid (80:20:1, v/v). Spots were visualized by spraying with sulphuric acid and heating at 110°C.

## RESULTS

Although it has been established (1–6) that lipids are major precursors to chloropropanols, previous studies have not ruled out carbohydrates as precursors. Pentosan (arabinoxylans) and pectin (mainly methyl-esterified galacturonic acid) react with hydrochloric acid to produce substantially lower yields of chloropropandiols than obtainable from crude soya meal or maize gluten, indicating that they are not the principal precursors (Table 1).

Maize glutens which have been extracted with chloroform/methanol (2:1) (to reduce the lipid content), react with hydrochloric acid to generate a lower yield of chloropropanols than nonextracted maize gluten. Hydrolysis of a mixture of extracted maize gluten and glycerol (equivalent to the level of glycerol present as glycerol esters) led to a lower (40%) yield of chloropropanols than obtained from crude maize gluten, suggesting that glycerol esters rather than glycerol are the precursors to chloropropanols.

Crude proteins used for the manufacture of hydrolysates contain a range of glycerolipids and phospholipids. Triolein and 1,2-diacyl-*sn*-glycerophosphorylcholine were used in this investigation as model substrates to represent these classes of materials. Equimolar amounts of triolein, 1,2-diacyl-*sn*-glycerophosphorylcholine (PC) and glycerol were treated with hydrochloric acid under conditions typical of hydrolysate manufacture, and yields of chloropropanols were determined (Table 2). Yields of chloropropandiols from triolein and PC were significantly higher than from glycerol (2.6 times and 1.8 times, respectively). Triolein gave a much higher 3MCPD/2MCPD ratio than that obtained from glycerol and PC, suggesting that the three substrates react by different mechanisms to form chloropropanols.

Hydrolysis of mixtures of glycerol (pentadeutero, d5)[1]

TABLE 1

**Reactions of Carbohydrates with Aqueous Hydrochloric Acid**

| Substrate | Substrate/HCl | HCl (molarity) | Reaction conditions | Total chloropropandiols (μg/g starting material) |
|---|---|---|---|---|
| Soya meal | 1:10 | 5.5M | Reflux/11 hr | 235 |
| Soluble pentosan | 1:10 | 5.5M | Reflux/11 hr | 5 |
| DM 33.7 pectin | 1:15 | 5.5M | Reflux/11 hr | 5 |

TABLE 2

**Reactions of Glycerol and Its Esters with Hydrochloric Acid**

| Substrate | Total chloropropandiols | Yield relative to glycerol | 3 MCPD/2MCPD ratio |
|---|---|---|---|
| Glycerol | 2.2% | 1 | 2.7 |
| Triolein | 5.7% | 2.6 | 10.1 |
| Phosphatidylcholine | 4.0% | 1.8 | 2.8 |

[1]Pentadeuterochloropropandiol could be resolved from chloropropandiol by capillary gas chromatography.

TABLE 3

**Reactions on Mixtures of Deuterated Glycerol and its Esters with Hydrochloric Acid**

| Substrate | Total chloropropandiols | Yield ratio d0/d5 | 3MCPD/2MCPD ratio | |
|---|---|---|---|---|
| | | | Glycerol-d5 | d0 |
| (d5) (d0) Glycerol + triolein[a] | 4.0% | 2.6 | 2.3 | 10.5 |
| (d5) (d0) Glycerol + PC[a] | 2.8% | 1.9 | 2.8 | 2.6 |

[a]Equimolar proportions.

TABLE 4

**Influence of Organic Acids on Reaction Between Glycerol and Hydrochloric Acid**

| Substrate | Total chloropropandiols | Yield relative to glycerol | 3MCPD/2MCPD ratio |
|---|---|---|---|
| Glycerol | 0.9% | 1 | 2.3 |
| Glycerol + acetic acid[a] | 4.1% | 4.6 | 6.4 |
| Glycerol + oleic acid[a] | 1.2% | 1.3 | 2.5 |
| Glycerol + glycine[a] | 1.2% | 1.3 | 2.5 |

[a]Equimolar proportions of glycerol and acids used.

TABLE 5

**Isomer Ratio of Chloropropandiols from Hydrolysis[a] of Soybean Meal with Hydrochloric Acid**

| Reaction time (hr) | 3MCPD/2MCPD ratio peak area |
|---|---|
| 2 | 4.0 |
| 4 | 5.1 |
| 7 | 5.2 |
| 16 | 4.9 |

[a]2 gm Soybean meal + 4 mL 5.5M HCl, 110°C.

with triolein or with PC gave a chloropropandiol composition for each substrate similar to that obtained from the hydrolysis of individual substrates (Table 3).

The effect of three organic acids on the formation of chloropropandiols from glycerol by reaction with aqueous hydrochloric acid was also investigated. Acetic acid was selected because of its solubility in water, oleic acid as a typical fatty acid present in phospholipids/glycerolipids, and glycine as an amino acid component of vegetable proteins. Acetic acid (1 molar equivalent) produced a substantial increase in yield (~five-fold) and an enhanced 3-chloropropandiol/2-chloropropandiol ratio. In contrast, both oleic acid and glycine produced a much smaller increase in chloropropandiol yield and 3-chloropropandiol/2-chloropropandiol ratio (Table 4). Clearly the presence of acetic acid changes the reaction mechanism by which glycerol and aqueous hydrochloric acid react to produce chloropropanols.

The composition of the chloropropandiol mixture obtained by the reaction of soybean meal with aqueous hydrochloric acid revealed a 3-chloropropandiol/2-chloropropandiol ratio of approximately five (Table 5). This differs from that obtained from the individual materials (triolein, PC and glycerol) investigated, and indicates that a mixture of lipid types and their hydrolysis products could contribute to chloropropandiol formation.

Dichloropropanols are present in protein hydrolysates at levels considerably lower than those of the monochloropropandiols (7). Since hydrochloric acid can provide only a single chlorine atom by substitution it is likely that the monochloropropandiols or their esters are the precursors to dichloropropanols. Hydrochloric acid produced a substantially higher yield (1000-fold) of dichloropropanol from 3MCPD under identical reaction conditions as glycerol. In accord with previous studies (*vide supra*), hydrolysis in the presence of acetic acid (molar equivalent) further enhanced the yield (approximately 20-fold), while the ratio of 1,3-dichloropropanol/1,2-dichloropropanol increased from 3.5 to approximately 10 (Table 6).

## DISCUSSION

The yields and product composition of chloropropanols obtained by the hydrolysis of glycerol and its esters with aqueous hydrochloric acid can be rationalized in terms of nucleophilic substitution by chloride anion. We have shown that yields of chloropropandiols decrease in the sequence: triolein > PC > glycerol, while the ratio of 3-chloropropan-1,2-diol/2-chloropropan-1,3-diol is similar for PC and glycerol, but different from that for triolein. Additionally, hydrolysis of a mixture of delipidated maize gluten and glycerol gives a lower yield of chloropropanols than obtained from crude maize gluten. Clearly PC and triolein do not form CP diols *via* prior hydrolysis to glycerol. The presence of reactive groups in close proximity to each other and in geometrical relationships ideally suited for intramolecular interactions results in mech-

TABLE 6

**Reactions[a] of Glycerol and 3-Chloropropandiol with Hydrochloric Acid**

| Substrate | 1,3-Dichloropropanol (%) | 1,2-Dichloropropanol (%) | Ratio 1,3DCP/1,2DCP |
|---|---|---|---|
| Glycerol | $< 7 \times 10^{-5}$ | $5 \times 10^{-5}$ | nd[b] |
| 3-Chloro-1,2-propandiol | 0.07 | 0.02 | 3.5 |
| 3-Chloro-1,2-propandiol + acetic acid (molar equivs.) | 1.56 | 0.16 | 9.8 |

[a]Substrates (~0.01 moles) hydrolyzed with 5.5M HCl (10 mL) @ 105°C/16 hr in a sealed tube.
[b]nd, Not determined.

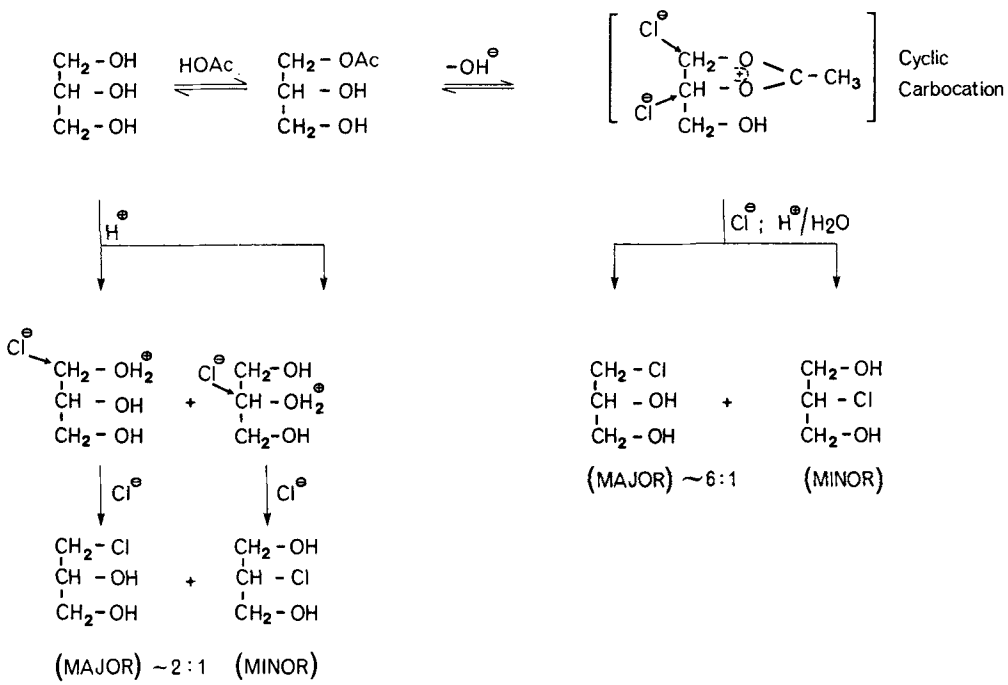


**FIG. 1. Formation of chloropropandiols from glycerol.**

anistic differences between the substrates and influences both yields and composition of isomeric products obtained.

The distribution of isomers (3-chloropropan-1,2-diol/2-chloropropan-1,3-diol, 2:1) obtained from glycerol is the result of nucleophilic substitution of the hydroxyl groups by chloride anion (Fig. 1). Although glycerol is soluble in aqueous hydrochloric acid and provides a high concentration of substrate for reaction, the low leaving propensity of the hydroxyl group results in a low yield of chloropropandiols. The isomer distribution is in accord with statistical substitution at two primary methylenes ($CH_2$) and one secondary methine group (CH).

In contrast, triolein has a low solubility in the aqueous reaction medium but reacts with hydrochloric acid to give a higher yield of chloropropandiols than obtained from glycerol; additionally, the regiospecificity is greater, with a 3-chloropropandiol/2-chloropropandiol ratio of approximately 10:1. The reaction can be explained as proceeding *via* the partial glycerides (1,3- and 1,2-diglycerides) with the ester group(s) providing anchimeric assistance through the formation of a cyclic acyloxonium ion intermediate (Fig. 2). Thin-layer chromatographic analysis of the reaction mixtures during the early stages of hydrolysis revealed the presence of both the 1,2- and 1,3-diglycerides. The ratio of chloropropanol isomers is controlled by the steric and electronic effects arising from the terminal ester group, which directs substitution to the $CH_2$ carbon atom.

The effect of acetic acid on the reaction of glycerol with hydrochloric acid supports the mechanism proposed for the triolein reaction. The observed increase in 3-chloropropan-1,2-diol/2-chloropropan-1,3-diol ratio from 2.5:1 to 6:1 is likely to have arisen from the *in situ* formation of glycerol monoacetate (monoglyceride) and its subsequent nucleophilic substitution by chloride anion (Fig. 1). The abundance ratio of 6:1 for the two chloro isomers is lower than that found for triolein (10:1), since the hydroxyl group at C-1 exerts a smaller steric and electronic effect at the methylene carbon than the ester

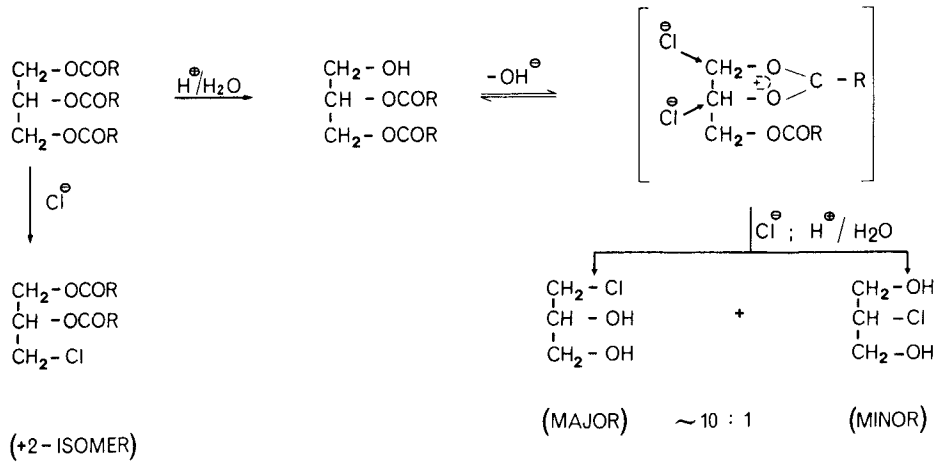


**FIG. 2. Formation of chloropropandiols from triglyceride.**

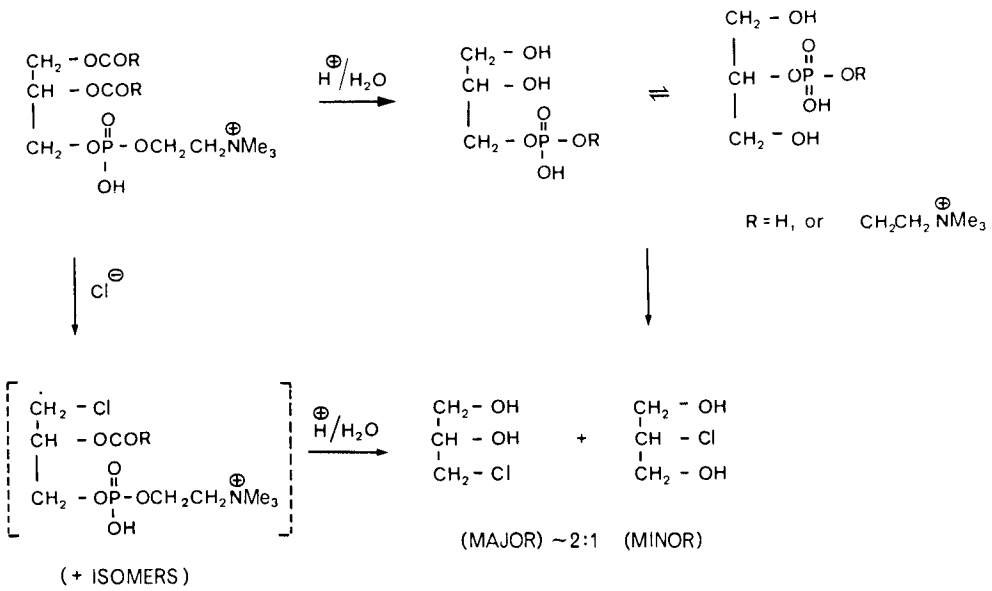


**FIG. 3. Formation of chloropropandiols from 1,2-diacyl-*sn*-glycero-3-phosphorylcholine.**

group. Oleic acid (which has low solubility in the reaction medium) and glycine (which is protonated in acid) both had little effect on yields and product composition, presumably because they were not able to react with the glycerol.

Although 1,2-diacyl-*sn*-glycero-3-phosphorylcholine generates a higher yield of chloropropandiol than glycerol, it gives a similar isomer distribution. Analysis of the reaction mixture by thin-layer chromatography during the early stages of hydrolysis revealed a substantial amount of fatty acid, but no 1-monoacyl-*sn*-glycero-3-phosphorylcholine. Hydrolysis to the totally deacylated derivative(s) (*sn*-glycero-3-phosphorylcholine or *sn*-glycero-3-phosphatidic acid) apparently had occurred. Phosphate and phosphorylcholine are more effective leaving groups than hydroxyl, but they exercise little regioselectivity (Fig. 3) due to facile intramolecular isomerization.

The enhanced yield of dichloropropanols from monochloropropandiol compared with that from glycerol shows that the former is the more likely precursor to dichloropropanols. The additional increase in yield when the hydrolysis with hydrochloric acid is carried out in the presence of acetic acid and the change in isomer distribution which occurs (1,3-dichloropropanol/1,2-dichloropropanol increased from 3.5 to 9.8) are again readily explained by the formation of a cyclic acyloxonium ion intermediate (Fig. 4).

These investigations have demonstrated that the yield and composition of chloropropanols formed during the hydrolysis of crude vegetable protein meal will depend on the composition of the lipid present in the meal. The reaction proceeds by nucleophilic substitution, while differences in yield and composition are governed by the

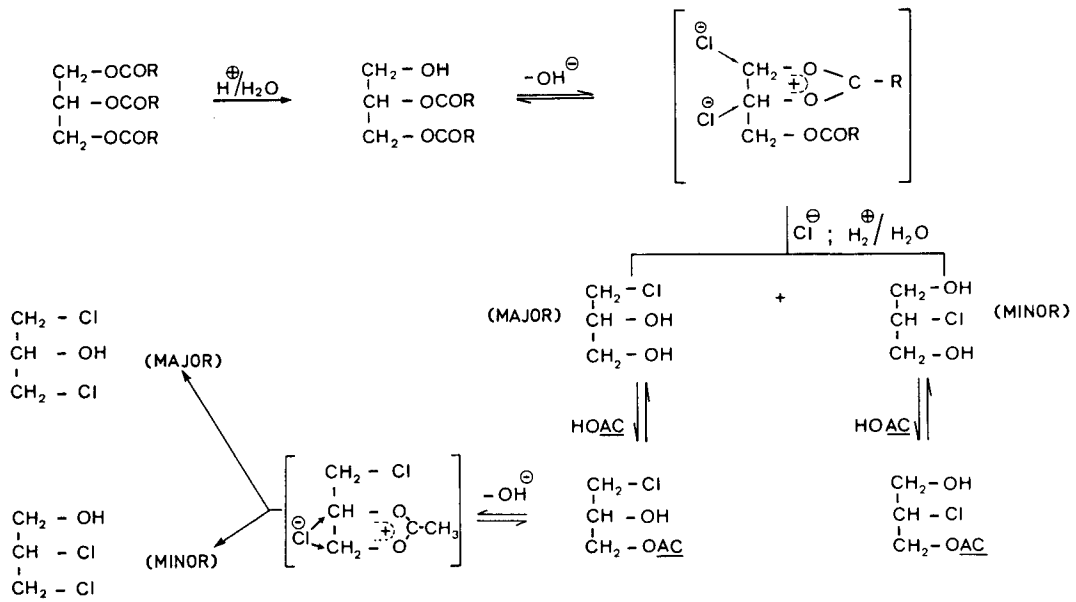


**FIG. 4. Formation of dichloropropanols from triglyceride.**

presence of neighboring ester groups that provide anchimeric assistance and control regioselectivity.

## ACKNOWLEDGMENTS

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