THE REACTION OF CHOLESTEROL WITH UNTREATED CHROMOSORB W DURING GAS CHROMATOGRAPHIC ELUTION

by

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REVIEW OF THE LITERATURE

Chromatography is an analytical separation method based on the differences in partition coefficients of substances distributed between two immiscible phases, one a static phase, usually of very high surface area, and a moving or mobile fluid phase. That this process has been active in nature since time immemorial is most likely; but its use as an analytical tool and scientific technique has a history of only a little over 50 years. The first reported experiments that can truly be regarded as chromatography are those of Tswett (61), who separated components of plant pigments by passing solutions of these materials through columns of solid adsorbents.

The next major step in the advancement of chromatography came with the work of Martin and Synge (44). Their reasoning that partition isotherms were more often linear for the partition of a solute between two liquids than for partition of a solute between a solid surface and a liquid led to construction and operation of a liquid-liquid system. For these contributions and for their finding that paper strips could be used in place of columns, they were awarded the Nobel prize in 1954, illustrating the tremendous impact of their contributions to all areas of chemistry.

As has been so often pointed out, it was in this original paper by Martin and Synge that they mentioned in passing that the mobile or flowing liquid could be replaced

by a gas. That no one picked up this suggestion is difficult to understand in light of the work done in the areas of frontal and displacement gas chromatography. In any event, ten years passed before the method was tested by James and Martin (34). This work by James and Martin marked the beginning of a period of development of elution gas chromatographic techniques that is almost unparalleled in any other field of science. The vast number of publications dealing with or utilizing this technique has led to development of an abstracting service for this field alone.

Elution gas chromatography has made significant contributions to three main areas. The widest application of this technique has been in the area of separation of complex mixtures. Secondly, preparative scale work has provided isolation of pure materials that had been impossible to obtain previously. The third application has been in the area of obtaining purely physico-chemical information. Work by Hoare and Purnell (24,25), for example, has pointed out how vapor pressures, boiling points, latent heats of solution and mixing, and activity coefficients may be measured.

The chemical basis for chromatographic separation lies in the differences of the partition coefficients of the materials to be separated. If two immiscible phases A and B are in contact with a solute which is soluble in both, the solute will be distributed between the two phases. The partition coefficient K is given by the expression

$$K = {}^{c_A}/_{c_B} = {}^{c_s}/_{c_m}$$

where c_s represents the concentration of the solute in the phase held stationary on the column and c_m represents the concentration of the solute in the mobile phase moving down the column. The value of the partition coefficient should be constant at a given temperature. The common method of presenting partition data is a plot of c_A vs. c_B called the partition or distribution isotherm. For ideal conditions this plot should be linear and have a slope K.

If some device is placed at the column outlet to monitor instantaneous concentration of the effluent stream, a resultant elution chromatogram with a symmetrical Gaussian shaped peak for each solute is obtained.

In some situations, however, ideal conditions are not met, the partition isotherm is not linear, and the resulting elution peaks do not have the desired symmetrical Gaussian shape. To most workers such peak asymmetry introduces an intolerable uncertainty into any analytical results; however, in many situations useful information may be obtained about the conditions that contribute to the asymmetry.

There are several conditions that may contribute to partition isotherm non-linearity. First, a non-linear isotherm results if the activity of the solute shows a concentration dependence that is different for the two states.

Second, a change in molecular state gives a non-linear isotherm. For example, the solute may be monomeric in one phase but completely dimeric in the other phase.

If the partition coefficient is expressed in terms of the concentration of the monomer, a non-linear isotherm results. Such a situation exists in the gas-liquid equilibrium of acetic acid. In the gas phase acetic acid is considered to be almost completely dimerized up to the boiling point. If a polar liquid phase is chosen, a situation with a monomer in the liquid phase and a dimer in the mobile phase would result, giving a non-linear isotherm. James and Martin (34) have suggested that this type of behavior may be responsible for the very asymmetric gas chromatographic elution peaks that are often obtained for free fatty acids.

The most common cause of peak asymmetry involves the interaction of the solute with the "inert" material used to support the liquid phase. Concerning the nature of tailing, Giddings (18) has suggested that tailing may result from adsorption of solute molecules on active sites on the support surface. According to this active site mechanism, peak asymmetry will result when an active site holds a molecule for a time equal to that required for 1/4 of the solute zone to pass by. The tailing portion of the peak will increase as more and more of the solute molecules interact with active sites.

There appears to be some relationship between the ability of a compound to form a hydrogen bond and this type of peak tailing. Generally, compounds containing hydroxyl groups display the strongest tailing. Compounds that contain more than one hydrogen bonding functional group show

particularly strong tailing characteristics.

It has been generally recognized that peak asymmetry becomes more severe with decreasing sample size. Also, a number of workers have shown that retention time generally increases with decreasing sample size for compounds that tail. Scholz and Brandt (57) have suggested that this phenomenon is due to adsorption by the support surface. If adsorption of the solute by the support surface gives rise to non-linear isotherm, different relative amounts of solute will be adsorbed for various sample sizes. The differences in amount adsorbed should give rise to different retention times for different sample sizes.

In other work Cremer and Huber (11) and Huber and Keulemans (32) suggest that the tailing edge of the chromatographic peak is related to the first derivative of the adsorption isotherm. In this work they were able to construct the adsorption isotherm from the tailing peak.

For these studies, a series of samples of varying size is injected onto the column, and a series of peaks is obtained. As shown in Fig. 1, the tailing edge of the largest peak covers the total range of concentrations, with each of the smaller peaks representing successively smaller portions of this tailing edge of the largest sample peak. As smaller and smaller samples are injected, the retention time becomes greater.

Generally, for a given adsorptive surface, the retention time will decrease with increase in sample size up to a certain point; after this, any increase in sample will give rise to a significant increase in peak height with the

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EXPLANATION OF PLATE I

Fig. 1. Tailing resulting from non-linear adsorption of solute.

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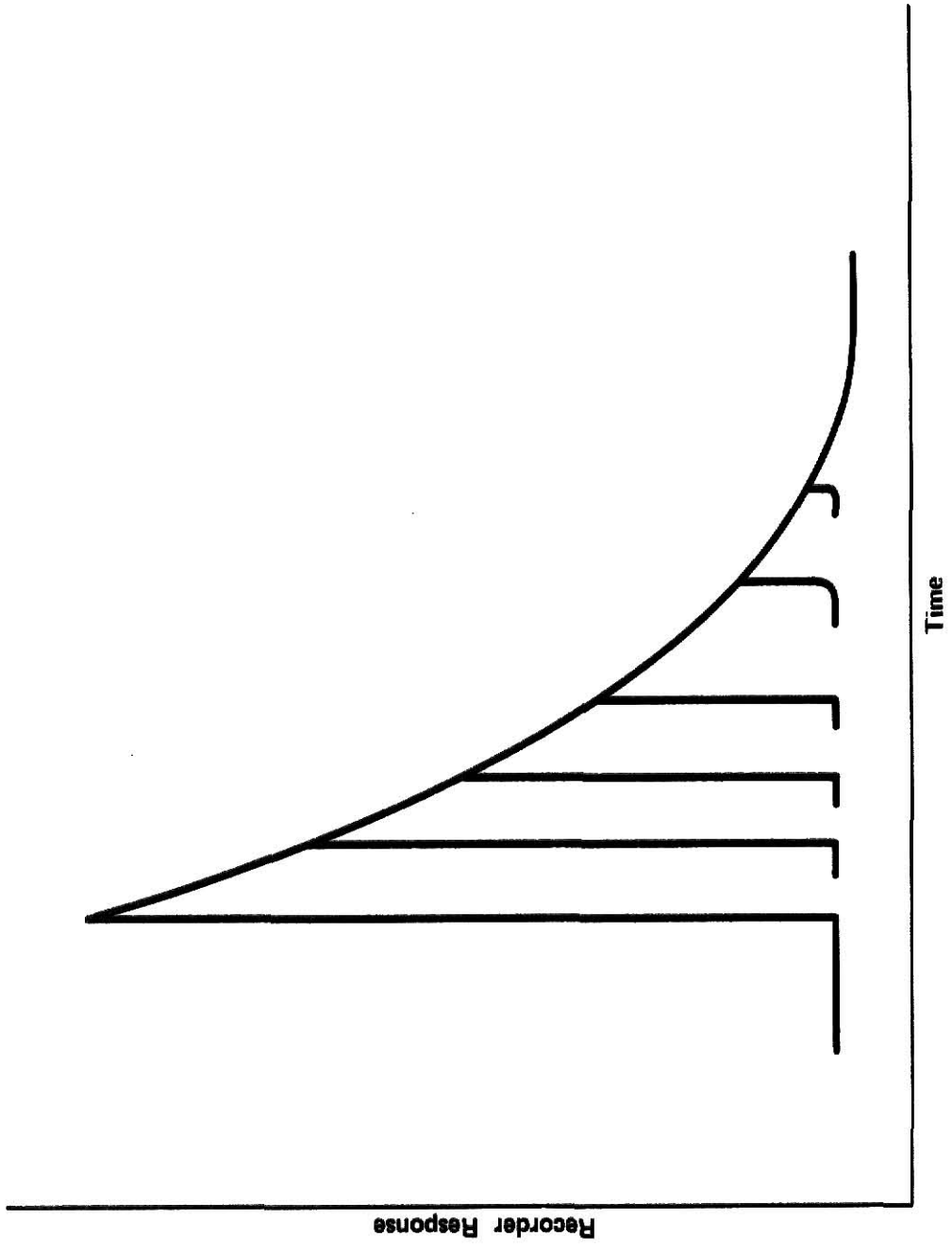
Plate I



retention time remaining constant. With this sample size enough solute material has been added to the column to saturate the active sites. Any amount above that required to cover the active sites will thus be seen as part of the elution peak, giving rise to a significant increase in peak height.

There is a third major class of solute interactions which can give rise to a tailing phenomenon and it is to this area that this research work is directed. The solute may undergo a chemical reaction during the gas chromatographic elution process. In some cases the reaction may take place very rapidly compared to the chromatographic time scale, giving rise to a relatively symmetric elution peak for the product. In many cases, however, the reaction takes place at such a rate that both products and reactants can be observed in the chromatogram. Several different situations are possible that give chromatograms showing characteristics of an on-column reaction.

For a simple reaction of solute A reacting to form product B, several chromatograms may be observed depending upon conditions and on the nature of the sample. If a pure sample of A is injected into the column and undergoes a slow irreversible reaction to form product B during elution, a chromatogram is obtained similar to that shown in Fig. 2. Depending upon conditions such as reaction rate and column length, more or less of the original solute A will pass through the column unchanged. Conditions could arise where

EXPLANATION OF PLATE II

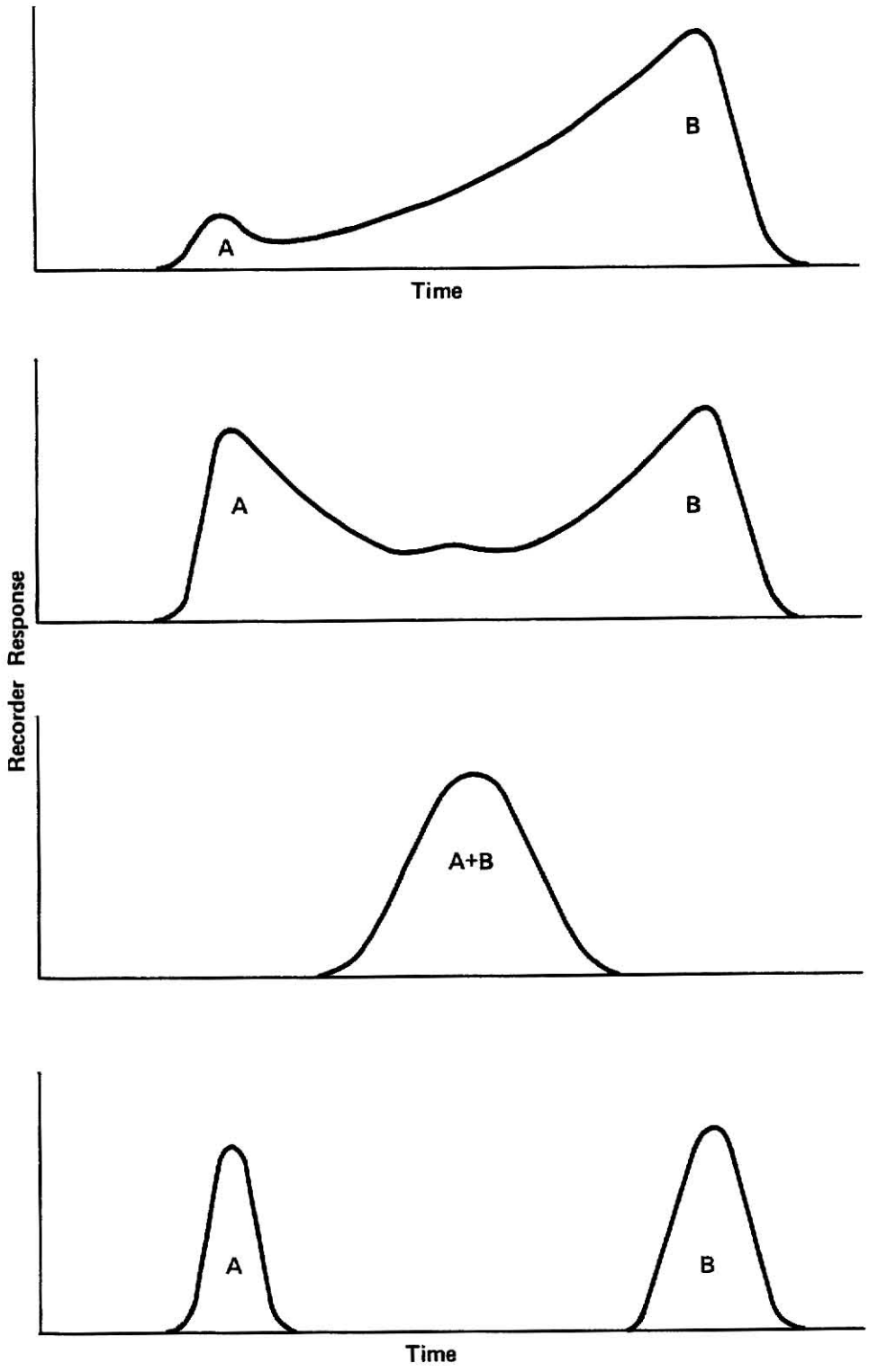
Fig. 2. Chromatogram for injection of pure A which undergoes a slow irreversible reaction to form product B during chromatographic elution.

Fig. 3. Chromatogram for injection of a mixture of A and B which equilibrates slowly on the column by a reversible reaction $A \rightleftarrows B$.

Fig. 4. Chromatogram for injection of a mixture of A and B which undergoes a fast reversible reaction $A \rightleftarrows B$.

Fig. 5. Chromatogram for injection of a mixture of A and B which does not react or which undergoes a very slow reversible reaction.

Plate II



A is completely converted to B giving rise to a tailing or fronting B peak that may resemble a peak that tails from adsorption.

For a reversible reaction $A \rightleftarrows B$, if a mixture of A and B is injected and equilibrates slowly on the column, a chromatogram is obtained similar to the one shown in Fig. 3. The slow reaction rate results in newly formed A and B being smeared over the column at relatively low concentrations. This results because A is formed primarily in the region of highest B concentration and B is formed in the region of highest A concentration.

If the reaction rate is fast enough that the probability of back reaction is significant with the result that the molecule makes several transitions back and forth between states A and B, the two peaks will merge into a single peak with has a retention time that is a weighted average of the retention times of the individual peaks. This is shown in Fig. 4. For a very fast reaction a single sharp elution peak would result. For a very slow reaction the two components should be separated and eluted, showing little or no tailing, Fig. 5.

Tailing resulting from an on-column reaction may be distinguished from tailing resulting from a non-linear adsorption or partition by injection of a series of samples of varying size. As shown in Fig. 1, a series of samples of varying sizes gives a series of peaks with coincident tailing edges if the tailing results from adsorption.

If, however, the tailing results from a chemical conversion of the solute during elution, the peak fronts, representing elution of unchanged solute, should coincide, as shown in Fig. 6.

There are numerous references in the literature to solute molecules undergoing a chemical conversion during elution. A selective sample of some of these reports may serve as a representative review.

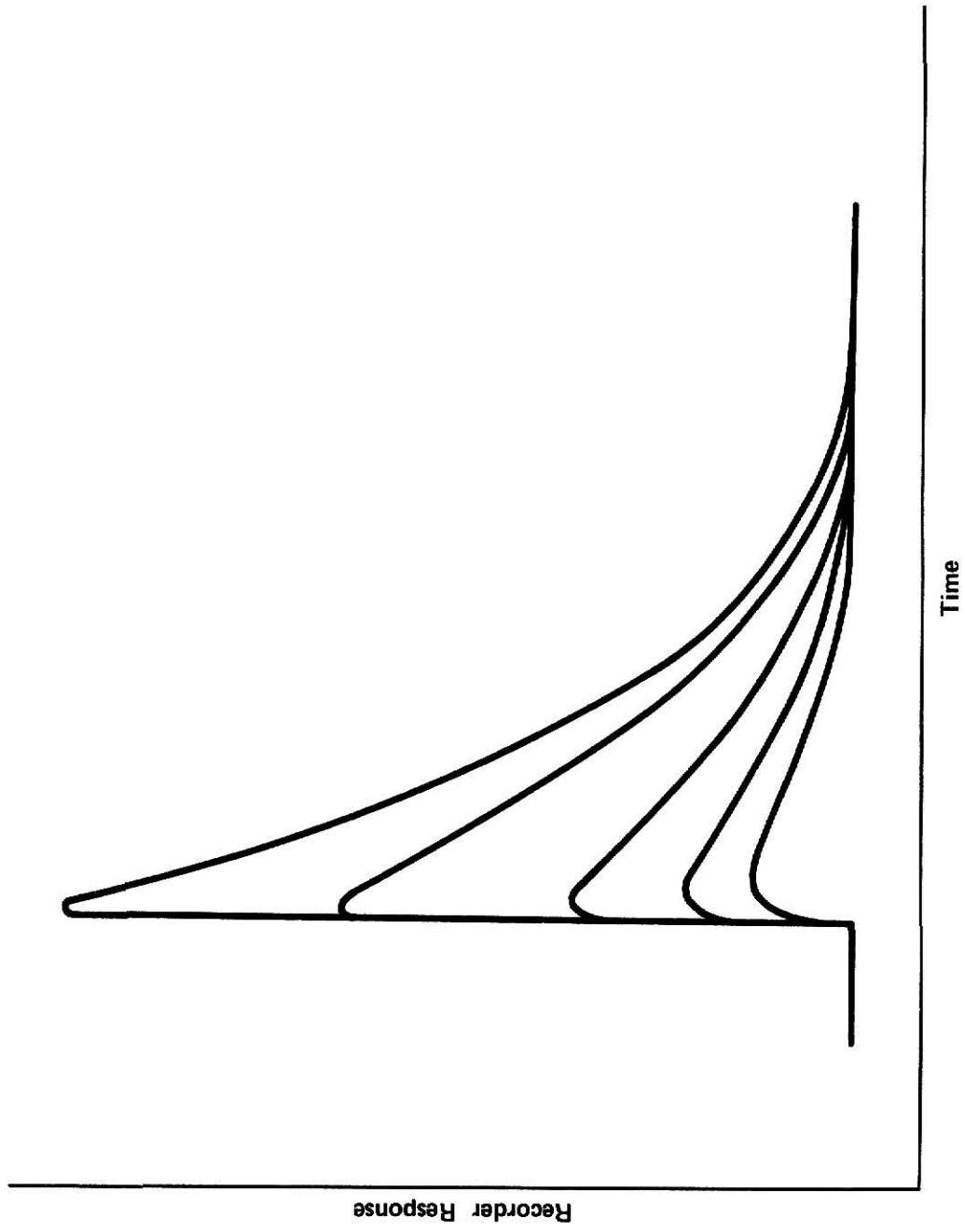
Many terpenoid materials appear to be very susceptible to isomerization and dehydration reactions. Although there are other factors such as acid contamination and excessive heat in the injection port that can catalyze these reactions, the support is generally considered to be responsible for catalysis. A discussion by A. Z. Conner (10) points out the catalytic decomposition of α-pinene to camphene on firebrick; acid contamination of the di-C_{10}-phthalate stationary phase produces the same effect. The catalytic effect of the active support material on terpene compounds has also been studied by Hayashi et al. (22). The entire problem has been reviewed in detail by Burchfield and Storrs (9).

Isomerization of conjugated allene and diene compounds catalyzed by the support material has been reported by Michel and Troyanowsky (45). Chromosorb P produced a strong isomerization; Chromosorb W was significantly less active. They also reported that even glass spheres show some activity.

EXPLANATION OF PLATE III

Fig. 6. Tailing resulting from a chemical reaction of the solute during elution.

Plate III



Morris et al. (47) have reported dehydration, deacylation and cis-trans isomerization of fatty acid methyl esters. The conversion of benzoin to deoxybenzoin and benzil catalyzed by the support has been reported by Bunbury and Osyany (8).

Steroid materials have also shown a sensitivity to diatomite support materials. Van den Heuvel (63) has studied the behavior of the methane sulfonates of 32 sterols which undergo an elimination reaction during chromatography. The structure of the olefin product is a function of the stereochemistry of the -OH group of the parent sterol and the presence of unsaturation and alkyl substitution in its immediate molecular environment.

Spencer (58) shows a chromatogram of cholesterol undergoing a dehydration reaction forming cholestadiene during chromatography on untreated Chromosorb W.

In almost all of these situations the reaction has been considered a nuisance that destroys the quantitative information. Several workers, however, have taken advantage of these reactions to extract kinetic information. The work of Bassett and Habgood (4) on the isomerization of cyclopropane catalyzed by molecular sieve is one of the earliest attempts to extract kinetic information. Although they recommend re-chromatography on a passive column, they point out that the relative amounts of the reactant and product may be determined by measurement of peak areas. If the assumptions of a first-order irreversible reaction and fast linear

adsorption of reactant independent of the presence of products may be made, the amount of product formed, as represented by the peak area, should be proportional to the rate constant. The contact time with the molecular sieve was measured as the retention time of the reactant.

In another early work Kallen and Heilbronner (37) discussed a theoretical treatment of the chromatogram of a solute undergoing a first-order irreversible decomposition during elution. Their treatment is based on the assumptions of a theoretical plant of finite dimension and conservation of mass. They show a number of calculated concentration profiles which show the expected exponential decrease in the amount of reactant with time.

The work of Keller and Giddings (38) and earlier work by Giddings (19) led to development of a model which allows prediction of sample distribution during elution from a reactive column, if conditions of linear ideal chromatography may be assumed. Correction for conditions of chromatographic spreading by assigning effective diffusion coefficients to each of the eluents allows calculation of concentration profiles from the kinetic and experimental parameters. Unfortunately, the model does not allow an easy calculation of kinetic parameters from observed chromatograms.

The work of van Swaay (66) suggests techniques for extraction of kinetic information directly from observed chromatograms. He points out that the mathematical expressions for both reversible and irreversible first-order reactions may be treated in closed form and allowances may be

made for non-ideal chromatographic conditions. Second order reactions of the type A + B→C are generally not encountered because the two reactants are separated shortly after injection. Reactions of this type may be reduced to pseudo-first-order by addition of a constant concentration of one of the reactants to either the mobile or stationary phase. Second order reactions of the type 2A→B do occur on chromatographic columns; they are, however, very difficult to treat mathematically.

For a first-order irreversible reaction taking place under conditions of linear ideal chromatography, van Swaay defines a new retention parameter υ^*

$$\upsilon^* = (\upsilon - \upsilon_B)/(\upsilon_A - \upsilon_B)$$

where υ_A and υ_B are the retention volumes of the reactant and product respectively. From a plot of ln S_x, where S_x is the detector signal at the retention volume x, versus υ^* the rate constant for the reaction may be extracted from the slope. An estimate of the range of time constants that may be studied is given; however, larger values of τ may be studied with the stopped-flow technique outlined by Phillips et al. (53). The model for linear-ideal chromatography may be corrected for non-ideal conditions by assuming that the total peak variance is equal to the sum of the chromatographic variance and kinetic variance. The total variance may be experimentally determined and the chromatographic variance may generally be predicted from the peak width of one or more non-reacting compounds. An expression for the kinetic variance, which may be

measured by difference, is derived in terms of the reaction time constant.

For a reversible first-order reaction the molecule may exist in two states A and B. If a suitable moving coordinate system is defined, a comparison to chromatography with a partition between a stationary state A and a moving state B may be made. In such a system the reaction rate is analogous to the mass transfer coefficient of conventional chromatography. Van Swaay has shown the relationship between H*, the height equivalent to a theoretical plant in the moving coordinate system, and the reaction time constant τ. An expression is given that allows the determination of τ from the kinetic variance contribution that may again be determined by the difference between total variance and the chromatographic variance of the elution peak. Rate constants of the order of 20 sec^{-1} may be studied by means of this technique.

The focal point of the present research was an attempt to apply the mathematical model developed by van Swaay to the dehydration of cholesterol to cholestadiene reported by Spencer (58).

It should be recognized that the interaction between cholesterol and the "inert" diatomaceous earth support is as much a function of the inert support as of the steroid solute. A brief look at the chemistry and gas chromatography of cholesterol and the nature of the diatomaceous earth support should contribute to the understanding of this interaction.

Before 1960 several questionable theoretical assumptions combined with unsuitable experimental conditions led most workers to believe that steroid materials with molecular weights of 250 to 400 and high melting points could not possibly be chromatographed at normal pressures, even at elevated temperatures.

A high melting or boiling point at atmospheric pressure does not dictate that the compound must have a long retention time, because, thermodynamically, it is the free energy of solution, not a high boiling point, that determines retention time of a solute. The boiling point of a pure substance is related to the energy required for the escape of molecules from the environment of similar molecules and for many compounds with one or more functional groups strong intermolecular forces must be overcome. In a gas chromatographic situation, however, interactions in the liquid phase are not between solute molecules, but rather between the solute molecules and the stationary phase. If a non-polar stationary phase is used, solute-solvent interactions are much weaker than might be anticipated.

At this time it was also believed that the elevated temperatures required for chromatography would result in thermal decomposition of the solute. It is indeed true that many steroids are destroyed when heated in air, but this is not a thermal effect per se; many organic materials are much more seriously affected by oxygen and light than by heat.

Thick-film columns with stationary phase loadings of at least 5% and common loadings of 20%-25% showed only a qualified success. It was necessary to use either excessively high temperatures to achieve a short elution time with the resulting short column life and thermal decomposition of some steroids and stationary phases or to go to lower temperatures with the resulting elution times measured in hours. When it was recognized that the supposed requirement of at least 5% stationary phase loading was not theoretically sound and that it was based on inadequate recognition of the practical problem of deactivation of the solid support surface, the separation of steroids and other compounds of relatively complex structure by gas chromatography became practical.

The problem was solved by Van den Heuvel, Sweely, and Horning (65) in 1960. They showed that low loaded columns (1-3%) could be prepared with thermally stable stationary phases and a properly deactivated support. With these columns they were able to separate a number of steroids without structural alteration and with retention times of 15 minutes to one hour. Proper support deactivation was the crucial work that allowed the low stationary phase loading and the resulting success. This support deactivation will be discussed in detail in a following section.

Several experimental complications are present in chromatography of these materials that are generally not found in chromatography of smaller and less sensitive molecules.

Materials conventionally used for columns, flash heaters, and connecting components frequently interfere with steroid analysis. Arnold and Fales (1) made a study of column and connector materials, finding that copper columns could not be used at all. Generally, basic materials were almost completely adsorbed on copper columns; a cholesterol peak for example, was greatly diminished in area compared to a standard cholestane peak. For an injection port these workers found glass to be slightly superior to aluminum or stainless steel and copper totally unsatisfactory; however, no pretreatment of the glass surface is mentioned. Teflon tubing appeared to cause considerable tailing and some loss of peaks of basis amines at 300°C. Most workers prefer glass columns and glass-lined flash heaters; some pretreatment is generally necessary to insure that no solute-glass surface interactions take place.

The major contribution to tailing peaks in gas chromatography, whether it be due to adsorption or chemical reaction, involves an interaction of solute with the "inert" solid support. The support material used in gas chromatography has the sole purpose of serving as a platform to hold the liquid phase in the column. The problem is that the inert support is now always inert.

In the original work by James and Martin (34), Celite 545, a filter aid, was used. This support was chosen primarily because of the author's experience with it in reverse phase partition chromatography. Most of the diatomite

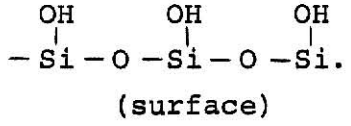
support materials now in use are derived from either diatomite firebrick or diatomite filter aids. Materials derived from diatomite firebrick are generally referred to as type I supports and those derived from diatomite filter aids as type II supports.

Type I support, which is commonly called pink Chromosorb or Chromosorb P, is derived from Sil-O-Cel C-22 firebrick; this firebrick is prepared from diatomite that has been crushed, blended, pressed into a brick and then calcined or burned above 900°C to allow its use as high temperature insulation. The type II support is commonly called white Chromosorb or Chromosorb W. This material is prepared from Celite filter aids which are made by mixing the diatomite material with a small amount of sodium carbonate flux and calcining at temperatures above 900°C.

Chemically the two types of diatomite supports are very similar except for the high Na_2O and K_2O content in the type II supports. The surface pH of the type I support is approximately 6·0 to 6·5, while the surface pH of the type II support is 8·0 to 8·5, reflecting the use of the sodium carbonate flux in preparation. Mineral impurities present may form complex oxides or silicates during the heating. Iron oxide is thought to give the characteristic pink color to the type I material.

Microscopic examination of type II supports indicates that the diatomite fragments may be held together with sodium silicate glass with much of the fine structure destroyed. Some of the silica is converted to cristobalite with type II support material; however, a white material results due to conversion of the iron oxides to colorless sodium iron silicate.

The chemical structure of the diatomite support surface is indeed responsible for catalyzing the reactions and for the adsorption that cause tailing. The diatomite is a siliceous material with up to 10% mineral impurities present. The surface of the siliceous material is covered with silanol groups (Si-OH) and siloxane (Si-O-Si) groups. The diatomite surface may be represented as



(surface)

Some mineral impurities are present at the surface, but it is not known in what chemical state they exist. Perrett and Purnell (52) studied the surface of type I and type II supports by reacting hexamethyl disilazane (HMDS) with the support surface, monitoring the reaction by-product, ammonia, and calculating the extent of reaction. They calculated that the type I support contains 4×10^{19} groups/m^2 and the type II support to have $2{\cdot}5 \times 10^{19}$ groups/m^2. It has been generally accepted that the type I support shows stronger adsorption characteristics than the type II support. This appears to be due only to a greater surface area per unit volume.

Perrett and Purnell (52) felt that their kinetic data indicate that there may be two types of active sites on the support surface. Papa (51) studied this proposal in more

detail using frontal analysis methods. He found that, indeed, two types of sites appear to exist, one of high and the other of lower activity.

Yates and Trebilcock (67) found two types of active sites on the surface of glass; one was the silanol group and the second was an acid group other than silanol. They proposed that this acid site may arise from isomorphous substitution of boron and aluminum for the silicon.

Undoubtedly the most important type of active site on the support surface is the silanol group. It may thus be expected that the most common type of support-solute interaction is hydrogen bonding. Numerous infrared spectroscopy studies of hydrogen bonding of the surface silanol groups have been made and are recorded in the literature.

A great deal of effort has been put forth to modify the support surface in order to reduce solute-surface interactions. There are five common techniques in practice and a discussion of these may offer a greater understanding of the support surface and its interaction with solute molecules. The five techniques are (1) saturation of the active sites with a liquid, (2) removal of the silanol groups by reaction, (3) removal of acid sites by washing with acid, (4) adjustment of surface pH, (5) coating the support with a solid.

The activity of the sites may be greatly reduced by introducing polar materials into the system, either into

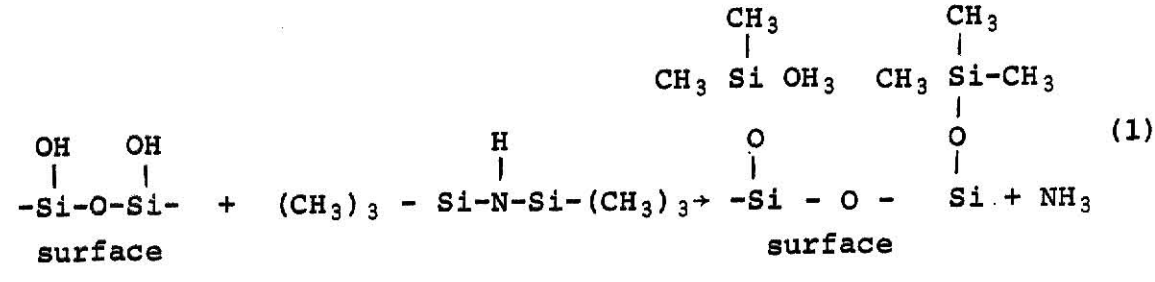
the carrier gas or into the liquid stationary phase. Although the mechanism by which this deactivation is accomplished is not totally understood, it appears that hydrogen bonding plays a significant role. Scholtz and Brandt (57) and Kuzy (42) predict that the deactivation is accomplished via a hydrogen bonding mechanism. These workers found that compounds that have high hydrogen bonding characteristics were very effective in deactivating the support surface, while compounds that are polar in that they have a high dipole moment, but that are not able to hydrogen bond, were not effective in deactivation. Thus, a common technique of support deactivation involves the use of a liquid phase that contains functional groups that hydrogen bond. A second technique saturates the carrier gas with a polar or hydrogen bonding material prior to entrance into the column. Water, ammonia, and alcohols are frequently used. Another technique involves saturation of the column with a sample which contains the compounds that tail. This is usually done by injecting a large volume of this sample prior to injecting the sample to be analyzed. With time, however, the material bleeds off the column and the column returns to its original state.

Liberti (43) suggested that active sites caused by mineral impurities on the support surface could be removed by acid-washing and/or base-washing. Chemical analysis of nonacid-washed and acid-washed supports shows

a small change in mineral content with acid-washing. Although the difference is small, it is probably significant since it is the surface that is most strongly affected by the washing. Horning, et al. (28,29) were the first to show definitely that the performance of a column could be improved by acid-washing the support. A cholesterol peak showed severe adsorption before the support was treated; after acid washing the cholesterol peak shape showed considerable improvement. Base washing the support seems to be of value only for the chromatography of amines. The value of this technique may be in changing the surface pH.

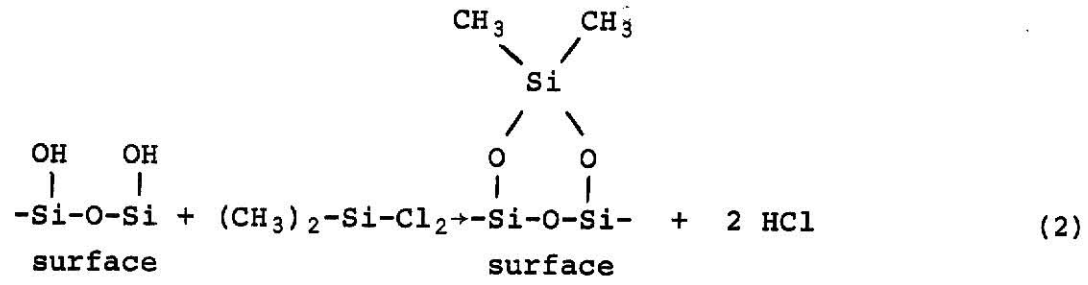
The most commonly used technique for surface deactivation and the technique that has been most effective in the area of steroid chromatography involves a reaction of the surface silanol groups. The most effective reactants have been silanes, particularly hexamethyldisilazane (HMDS) and dimethyldichlorosilane (DMCS).

The reaction of HMDS with the surface silanol groups is shown in Eqn. 1.



The surface silanol groups are converted to trimethylsilyl ethers with ammonia being given off as a by-product.

The reaction of DMCS is more complicated than the HMDS with two reactions possible as shown in Eqns. 2 and 3.



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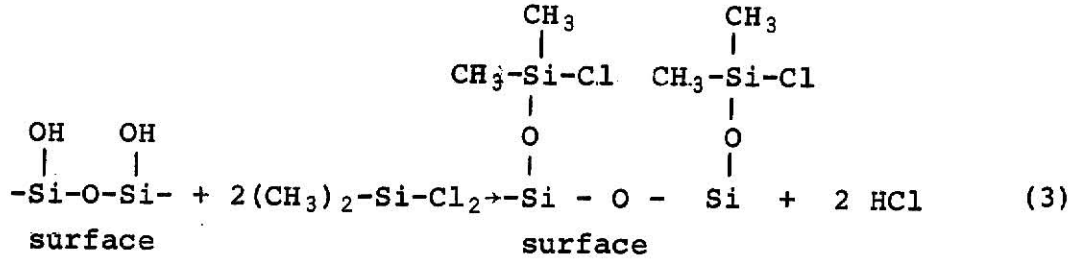

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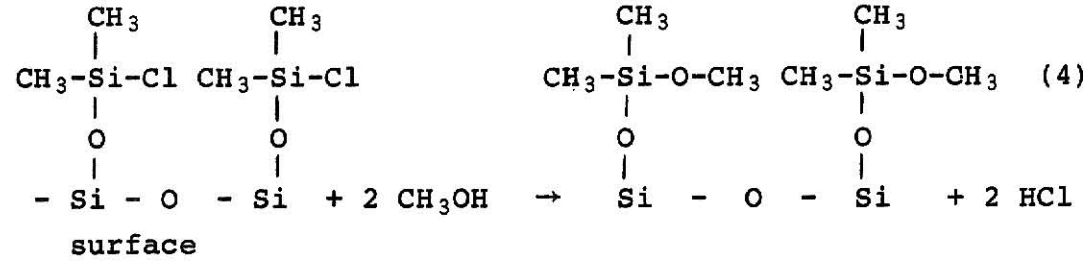


(4)

As can be seen the reaction may follow either of two plans; it is likely that both reactions take place. The reactive chlorine in the chlorosilyl ether must be removed by washing with methanol, Eqn. 4. This washing is necessary not only to convert the chlorosilyl ether but also to insure the removal of the HCl produced. If all the HCl is not washed from the column, the effectiveness of the technique is greatly reduced. The DMCS treatment is based on the work of Howard and Martin (31); they prepared a hydrophobic support for reverse phase chromatography by treating Hyflow Super-Cel.

The work of Horning et al. (28) describing a procedure for DMCS treatment of diatomite supports to be used for steroid chromatography was the stepping stone that allowed this area of analysis to develop.

Bohemen et al. (7) first described the use of HMDS for support deactivation, carrying out the reaction in petroleum ether under reflux conditions. An interesting technique using HMDS has been reported by Atkinson and Tuey (2). They reported an effective reduction in adsorption effects by treatment of the support in situ with HMDS. 50-µliter charges were injected directly into the column at 80°C with a low carrier gas flow-rate. This technique can not be used in cases where the liquid phase reacts with HMDS. In situ silanization has been a particularly useful technique for column rejuvenation; the HMDS not only covers active sites

that have been exposed with column use but also reacts with the non-volatile contaminations that may be present, forming volatile silane derivatives that are eluted off the column. DMCS in situ treatment is not recommended since the HCl produced causes some undesirable side effects.

Several studies have been made comparing the two reagents. Kabot (36) studied HMDS- and DMCS-treated columns used for steroid analysis and found that the HMDS-treated column needed further deactivation to give good peak symmetry with cholesterol. Kirkland (40) also found that DMCS-treated supports give better peak symmetry than supports treated with HMDS and trimethylchlorosilane (TMCS).

The temperature stability of the silane treated surface has not been determined; however, the work of Gavrilova et al. (16) reports that the $-O-Si-R_3$ groups are stable on silica gel up to 360°C. Although neither DMCS nor HMDS treatment of support material will give a totally inactive surface, the DMCS treatment is generally considered better than HMDS due to greater reactivity of DMCS. At high liquid loadings not much difference is apparent; however, at the low liquid loadings (<5%) commonly used for steroid analysis the DMCS treated support is definitely superior.

DMCS treatment combined with acid washing provides the best deactivation technique for the diatomite surface. It is generally difficult to see the effect of acid washing alone due to the large number of silanol groups; however,

when the silanol groups are masked, the difference between acid washed and non-acid washed supports becomes apparent.

The fourth method of surface modification involves the adjustment of the pH of the support surface. There are numerous literature references to compounds undergoing isomerizations and other acid or base catalyzed reactions during gas chromatographic elution. Treatment of the support with acid or base, as the case may be, generally has solved the problem.

The fifth form of support deactivation, coating the surface with a solid, has shown moderate success but has not found much popularity. The effectiveness of the technique lies in the ability to obtain a complete coverage of the surface. The first report of this technique was made by Omerod and Scott (48) on their work of coating a type-I support with silver and gold. The technique worked moderately well with silver. Onaka and Okamoto (49) coated both types of supports with Teflon, reporting good success in chromatographing highly polar compounds.

A much more complete review of support surface treatment has been given by Ottenstein (50); 200 references are listed.

EXPERIMENTAL TECHNIQUES

The Gas Supplies

The carrier gas (N_2) and the fuel gases (hydrogen and air) were supplied to the chromatograph via 100 psi precleaned plumbing. All gases were passed through a high pressure filter packed with 13 X Linde Molecular Sieve.

The Gas Chromatographic Column

To insure as much as possible that the cholesterol molecule would not undergo any reactions not catalyzed by the support, precautions to prevent reactivity of the remaining elements of the chromatographic system were taken. The material of choice for the chromatographic columns is Pyrex glass. Columns were made from 4mm i.d. Pyrex glass tubing. Columns of approximately 3, 6, and 9 feet were formed to fit the HP 700 Gas Chromatograph. The columns were constructed in such a manner that, if the standard injection port liner of the HP 700 is removed, the injection port end of the glass column can be passed up into the injection port to within 1cm of the septum. Thus the sample can be injected into a glass lined injection port and passed directly to the column without coming in contact with metal flash heaters or connecting materials. To further insure the inertness of the column, the glass surface was treated with a 10% (v/v) solution of dimethyldichlorosilane (DMCS) in toluene as outlined by Eik-Nes and Horning (13).

The column is filled with a 5-10% solution of dimethyldichlorosilane in toluene. After 15-20 minutes the column is emptied, thoroughly washed several times with methanol, and air dried.

The glass wool used for column plugs was also treated in a similar manner except that the glass wool was oven dried at 80°-100°C before use. Suitable precautions (plastic gloves and good ventilation) should be taken when dimethyldichlorosilane solutions are handled.

Support

The support material purchased for this study was 100/120 mesh non-acid washed Chromosorb W. This material was portioned and the various portions were treated in various manners.

Acid Washing and Silanizing the Support

A portion of the support was acid washed according to the procedure of Eik-Nes and Horning (13). To the flask containing the support material, concentrated HCl was added to cover the support and the mixture was allowed to stand for 24 to 48 hours. The HCl was then decanted or removed with a filter stick, after which fresh HCl was added. After one hour this HCl was decanted and the procedure repeated two or three times. The support was then washed well with deionized water. This should be done by decantation; the

fines may be removed at the same time. The support material was then separated off by filtration, washed well with acetone, spread out on filter paper to dry at room temperature for 15 minutes, and then oven dried over night at 100°-110°C.

A 10% (v/v) solution of dimethyldicholorosilane (DMCS) in toluene should be prepared in advance. Immediately upon removing the support from the drying oven (after the acid-washing procedure) the warm support was added to the DMCS solution in a side arm filter flask. The pressure was reduced and the flask swirled to dislodge any trapped air bubbles. The mixture was allowed to stand for 15 to 30 minutes. The support was then filtered off and washed on the filter with an equal volume of toluene. After this washing the support was slurried on the filter with methanol (300 ml methanol/25 grams of support). A second washing with methanol was made before air drying the support for 15 minutes at room temperature. The support was then oven dried at 100°C-110°C for two to three hours.

Coating the Support with SE-30 Stationary Phase

The SE-30 stationary phase was coated onto the various portions of Chromosorb W support material according to the procedure given by Henly, Kruppa, and Smead (23). For preparation of the low loaded column packing (<3%) required for chromatography of steroids this method of coating the support and, particularly, fluidized drying offer

several significant advantages. Parcher and Urone (62) discussed the technique of fluidized drying the coated support. They claimed that this method has several distinct advantages; (1) a high assurance of producing a uniform coating, (2) a definite savings in time, (3) less fragmentation of the support, and (4) expulsion of the fine particles during the fluidizing process. Considering these claims Kruppa et al. (23) compared the column efficiency of columns packed with packings prepared with fluidized drying techniques with the efficiency of columns packed with packings prepared by the most commonly used conventional technique-tray drying without stirring. They reported that packings dried by fluidization produced columns (6 foot) which averaged 1700 theoretical plates better than the columns made with tray dried packings. Kruppa states, "Since conversion to fluidized drying in this laboratory, 6-foot columns exhibiting 4000 plates or more for cholesterol have been frequently obtained with 3% loadings of SE-30, XE-60, and JXR on Gas Chrom Q. Before adoption of this technique, efficiencies of this magnitude were never encountered." A fluidizer similar to those described in these papers was fabricated in our shop.

A procedure very similar to the one outlined in Technical Bulletin No. 2A, Applied Science Laboratories, Incorporated, 1967, was used to prepare a column packing

of 3% SE-30 on the various portions of the 100/120 Chromosorb W support. The filtration technique in combination with fluidized drying procedure was used. The symbols used to calculate the amount of materials to be used are defined:

X = weight of the support to be used,

Y = W/V% stationary phase in solution divided by 100

To prepare a column packing, weigh X grams of the support into a container. Weigh 5XY grams of the stationary phase in another beaker and add 5X ml of the proper solvent to dissolve the stationary phase. Generally Y% (W/V) solution of stationary phase will give a Y% (W/W) coating on the packing. There are several exceptions to this condition, notably the silicone gums such as OV-1, JXR, SE-30, and UCW-98. For these materials a Y% (W/V) solution will generally give a 2Y% (W/W) coating; that is, a 1·5% (W/V) solution of SE-30 will give a 3% (W/W) coated support. Another factor which may be variable is the solvent to support ratio. For mesh fractions of 80/100 or higher, a solvent to support ratio of 5 is used; for mesh cuts below 80/100 this ratio should be changed to 6.

The stationary phase should be completely dissolved in the solvent; care should be taken to minimize evaporation of the solvent. When the stationary phase is completely dissolved, the support is slowly added to the solution with constant but gentle stirring and allowed to settle for 5

minutes. The material is then slurried again with gentle stirring and immediately poured onto a Buchner funnel mounted on a vacuum filter flask. With a water aspirator the excess solution is filtered off until dripping of the solution stops.

The damp packing material is carefully transferred to the fluidizer by means of a wide mouth funnel. The flow of preheated gas through the fluidizer is carefully adjusted; the wet solid plug of material should rise approximately 1/2 inch in the barrel. Care should be taken so that the pressure is not increased too rapidly, blowing the solid plug of wet packing material out the top of the fluidizer. After several minutes the packing should begin to fluidize. The fluidization must not be too violent. When the gas flow rate through the fluidizer is properly adjusted, the packing should look like a gently boiling liquid. As the solvent volatilizes off, a very cool region is evident on the barrel of the fluidizer. Fluidization is complete when the entire length of the barrel becomes warm. The material may then be poured from the fluidizer, the unit cooled, taken apart, and cleaned.

Sample Calculations

To prepare: 30 grams of 3% SE-30 on 100/120 Chromosorb W

X = 30 grams 100/120 Chromosorb W

5X = 150 ml $CHCl_3$

$$Y = \frac{1.5\%}{100} = 0.015$$

$$5XY = 150\ (0.015) = 2.25 \text{ grams of SE-30}$$

Packing the Glass Column

With plastic gloves, a small sample of DMCS treated glass wool is formed into a plug and inserted into the detector end of the DMCS treated glass column. This end of the glass column is then attached with rubber tubing to a suction flask trap which in turn is attached to an aspirator. The column is then clamped in a safe position with a small three finger clamp. The injection port end of the column is connected to the single outlet side of a two-way glass stopcock. One input side is connected to a funnel containing the coated packing material and the other inlet is left unattached.

To pack the coiled glass column, the pressure in the glass column is reduced by the aspirator; the two-way stopcock should not be opened to either inlet. With the pressure reduced in the column the two-way stopcock is opened to allow a "plug" of packing to enter the column. The stopcock is then closed and the pressure in the column reduced. The stopcock may then be turned to the other inlet side, allowing a "plug" of air to enter, forcing the "plug" of packing to the end of the column. Alternate "plugs" of air and packing material are introduced and the

packing material is continuously vibrated into place with a rubber tipped vibrating tool. The column should be filled with the packing only up to a point approximately three to four inches from the back of the injection port. Thus the coated column will not be affected by the very high injection port temperatures.

When the column is packed, the stopcock should be turned to the position open to the atmosphere and the aspirator disconnected. As before, a DMCS treated glass wool plug should be inserted into the injection port end of the column and pushed back against the packing with a wire rod. The column may now be inserted into the chromatograph and conditioned.

Connecting the Column

The problem of glass to metal connection at the elevated column oven temperatures was simply solved. A small silicone rubber "O" ring-type sleeve was cut from a sheet of septum material by means of the appropriate sized cork bores. The end of the glass column is inserted into a common Swagelok union, and the silicone rubber sleeve is pushed up to the union followed by a back ferrule with the flat band toward the sleeve. The Swagelok nut is tightened to a finger tight position. This unit provides a gas tight connection at 20 psi and 340°C for prolonged periods.

Cholesterol Sample Handling

A commercial sample of cholesterol was purchased. Because some halogenated solvents such as carbon tetrachloride and chloroform catalyze the decomposition of cholesterol to a black tar in the high temperature environment of the injection port (26), some care must be used in selecting a suitable solvent. The solvent of choice for the present work was benzene; hexane and isooctane are also suitable.

The most convenient sample solution was found to be a 10% (W/W) solution of cholesterol in benzene (thiophene free). With this solution sample sizes of 1-2μl are convenient.

The cholesterol sample must be injected into the chromatograph by the sample plug injection technique. The plunger of the syringe is drawn up several μls. The needle is inserted into the sample solution and a 1-2μl sample is drawn up. The needle is then withdrawn from the sample solution and the sample is drawn up into the barrel of the syringe surrounded on both ends by air. The needle is then carefully inserted through the septum into the glass column, the sample is injected with a smooth delivery of the plunger, and the needle is withdrawn.

This technique offers two definite advantages. First, with an air plug on each side of the sample, the sample volume may be accurately read. The entire sample

is contained in the barrel and there is no uncertainty about the amount of sample in the needle bore. The second advantage is that there is no sample in the needle bore at the time of insertion of the needle through the septum or after the injection has been made. If any sample is present in the needle for any period of time while the needle is at the elevated injection port temperatures (340°C), the benzene solvent is volatilized away and the cholesterol crystallizes in the needle bore. This results in a very seriously plugged needle.

Observing the Cholesterol Reaction

Beginning with a column oven temperature of approximately 210°C, cholesterol samples were injected onto columns thought to catalyze a cholesterol reaction. The column oven temperature was increased in 20°C increments until a temperature of 290°C was reached. At this temperature a significant cholesterol reaction was observed for a cholesterol elution time of approximately 15 minutes. At this temperature (290°C) a series of chromatograms was taken for various carrier gas flow rates. Varying the carrier gas flow rate varies the amount of time that the cholesterol sample spends on the column and thus varies the degree to which the reaction proceeds. It was necessary to use a stop-flow technique to obtain chromatograms in which the reaction proceeded to near completion.

A series of stop-flow chromatograms was run for various interrupted flow periods and oven temperatures.

Trapping Reaction Products and Reactant

Because a flame ionization detector was used for the present work, it was necessary to construct a splitting valve to be placed between the column and the flame ionization detector. The splitting valve, Figs. 7,8 is constructed in such a manner that under normal chromatographic conditions the column effluent passes directly through the valve to the detector. If, however, a peak is to be split off and trapped, the plunger is simply withdrawn from the valve barrel. The column effluent then passes out through the splitting valve into the trap. By placing a restriction in the tubing connecting the splitting valve and the flame ionization detector, the splitting ratio (the proportion of the effluent passing out into the trap compared to proportion passing through to the detector) can be adjusted.

The exhaust port of the splitting valve leading to the trap is tooled to the shape of a 10/30 inner connector; thus, glass traps with 10/30 outer connectors can be attached directly to the splitting valve. The splitting valve is fabricated from stainless steel to insure an inert surface and minimize the possibility of any sample reaction. The valve is mounted inside the column oven and thus kept at column temperature, eliminating any problem of sample condensation in the valve. The low dead volume of the

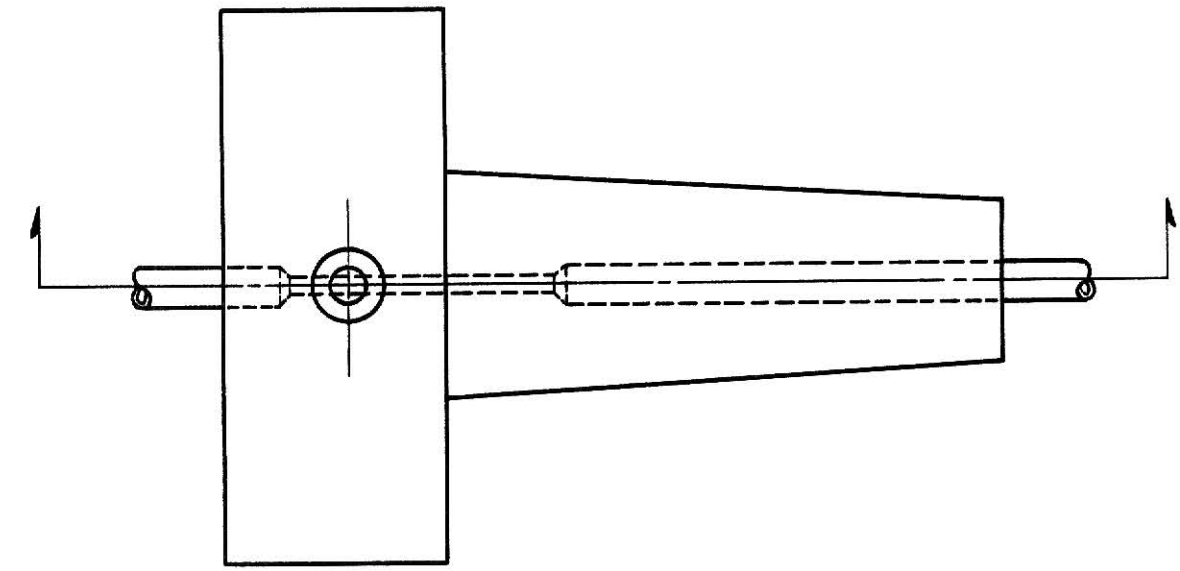
EXPLANATION OF PLATE IV

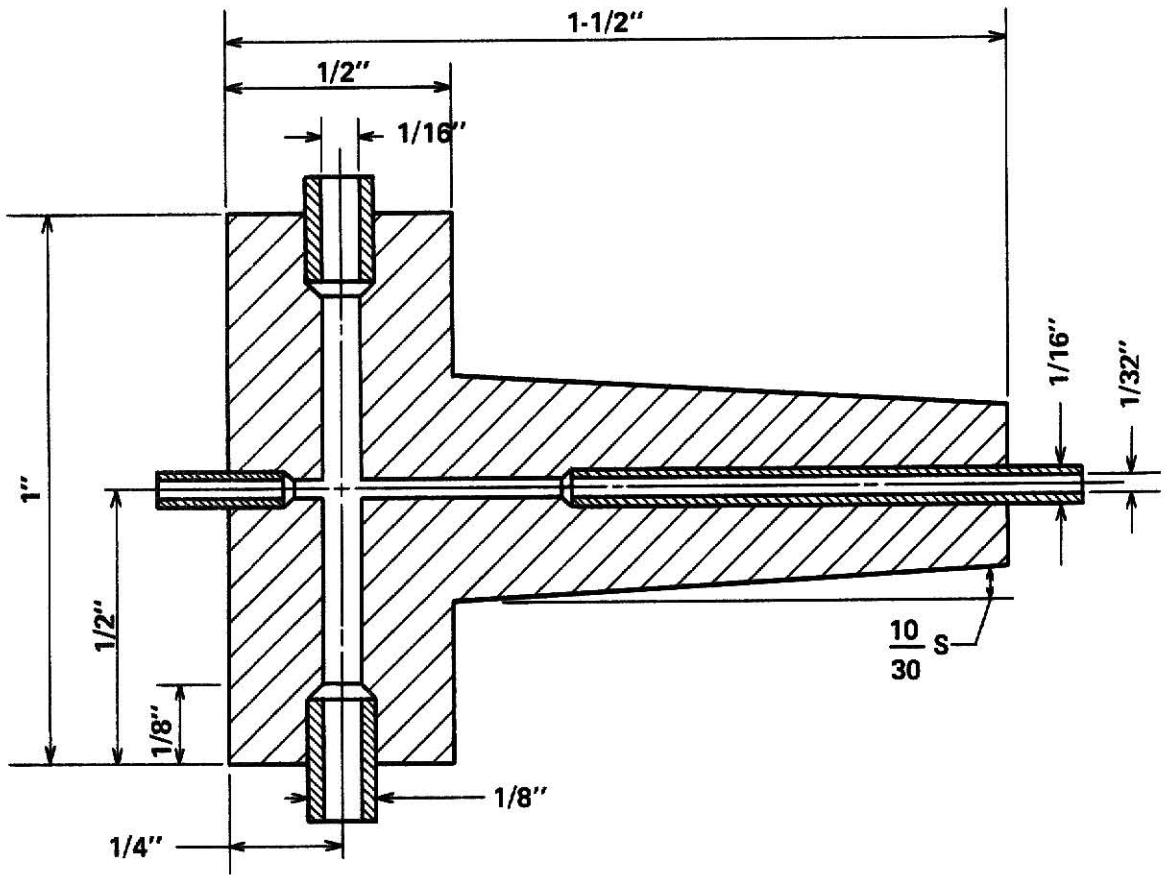
Fig. 7. Diagram of stainless steel splitting valve.

Plan View

Section View

Plate IV





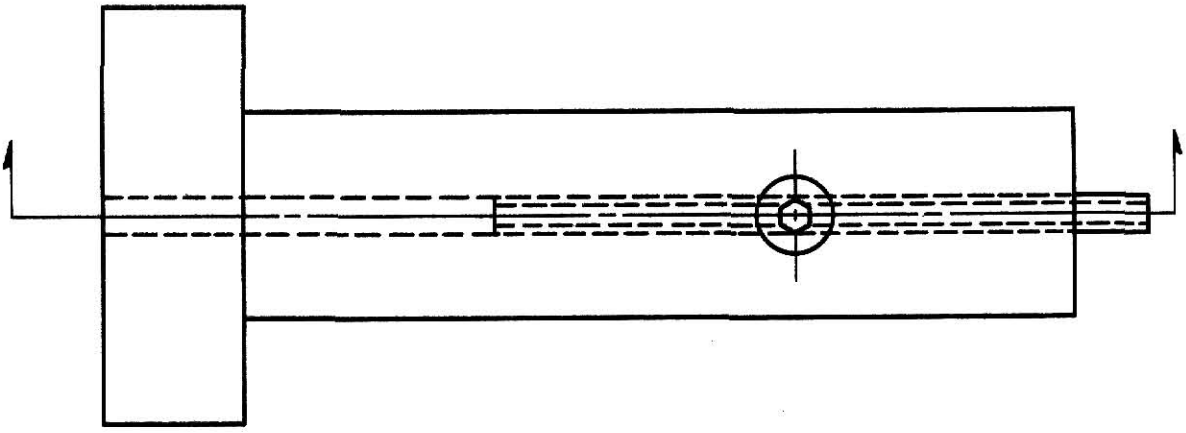
EXPLANATION OF PLATE V

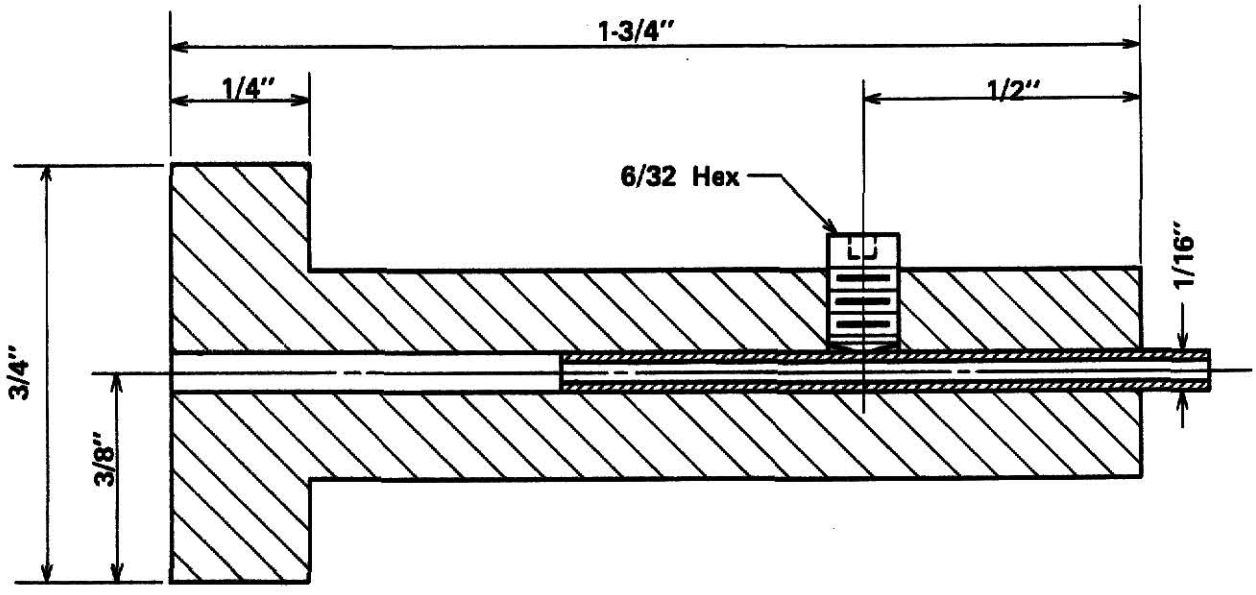
Fig. 8. Diagram of splitting valve plunger guide fabricated from a common iron stock.

Plan View

Section View

Plate V





valve (60µl) easily allows the trapping of a single elution peak or even portions of a single peak.

Simple glass traps were made to collect the products and reactant. A 10/30 outer glass connector is joined to a 20cm length of thickwalled capillary tubing. The entire glass trap is treated with dimethyldichlorosilane and methanol exactly as the glass columns. This glass trap is passed through a small hole in the column oven wall and connected to the stainless steel splitting valve. The trap is long enough that approximately 15cm of the capillary tubing extends out beyond the oven wall. It had been previously determined that all the products and the reactant condense on the capillary tubing wall at room temperature. Each product and the cholesterol reactant was trapped in its own glass capillary tube, the tube sealed, and the sample saved for subsequent analysis.

From the experiments studying the reaction rate, the optimum conditions for trapping each of the elution peaks were chosen. Because the diatomite support material was shown to be responsible for catalyzing the reaction, a pre-column was packed between the injection port and the normal chromatographic column to increase the product yield. This three foot pre-column was packed with uncoated and untreated 100/120 Chromosorb W.

If the rate of product formation was still low, a stop-flow technique was used. The cholesterol sample was

injected and allowed to reach the pre-column, the carrier gas flow stopped, and the reaction allowed to proceed. After a time the carrier gas flow was returned to normal, the components eluted, and the appropriate peak split off and collected.

RESULTS

Attempts to reproduce the dehydration of cholesterol to cholestadiene at the conditions reported by Spencer in Instrumentation in Gas Chromatography, J. Krugers, editor, proved to be totally futile. The conditions for this study were:

Column: 6ft. X 4mm ID DMCS treated glass
Packing: 3·8% SE-30 on 100/120 mesh (naw) Chromosorb W
Column Temperature: 230°C isothermal
Detector: flame ionization detector
Sample: 1-2μl 5% cholesterol in benzene.

For all the sample injections made chromatograms were obtained showing only a single elution peak. One chromatogram was taken out to 90 minutes past injection to insure that no compounds with long elution times were present. Generally, the shape of the single elution peak was very close to Gaussian, showing only a small amount of tailing. With injection of smaller samples, however, chromatograms showing greater tailing of this single elution peak were obtained. Because the tailing became much more severe with decreasing sample size, it was suspected that this tailing resulted from non-linear adsorption rather than from an on-column reaction.

To confirm this suspicion, a series of various sized samples of .1% cholesterol in benzene was injected onto a column of 4% SE-30 on 100/120 non-acid washed Chromosorb W at 240°C. If the tailing results from non-linear

adsorption, this series of samples should give rise to a series of elution peaks with coincident tailing edges, as shown in Fig. 1. If, however, the tailing results from a chemical conversion of cholesterol during elution, the peak fronts, representing the elution of unchanged sample, should coincide as shown in Fig. 6. The series of relatively small samples gave a series of elution peaks with very nearly coincident tailing edges, Fig. 9, indicating that this tailing results from non-linear adsorption of the eluting species.

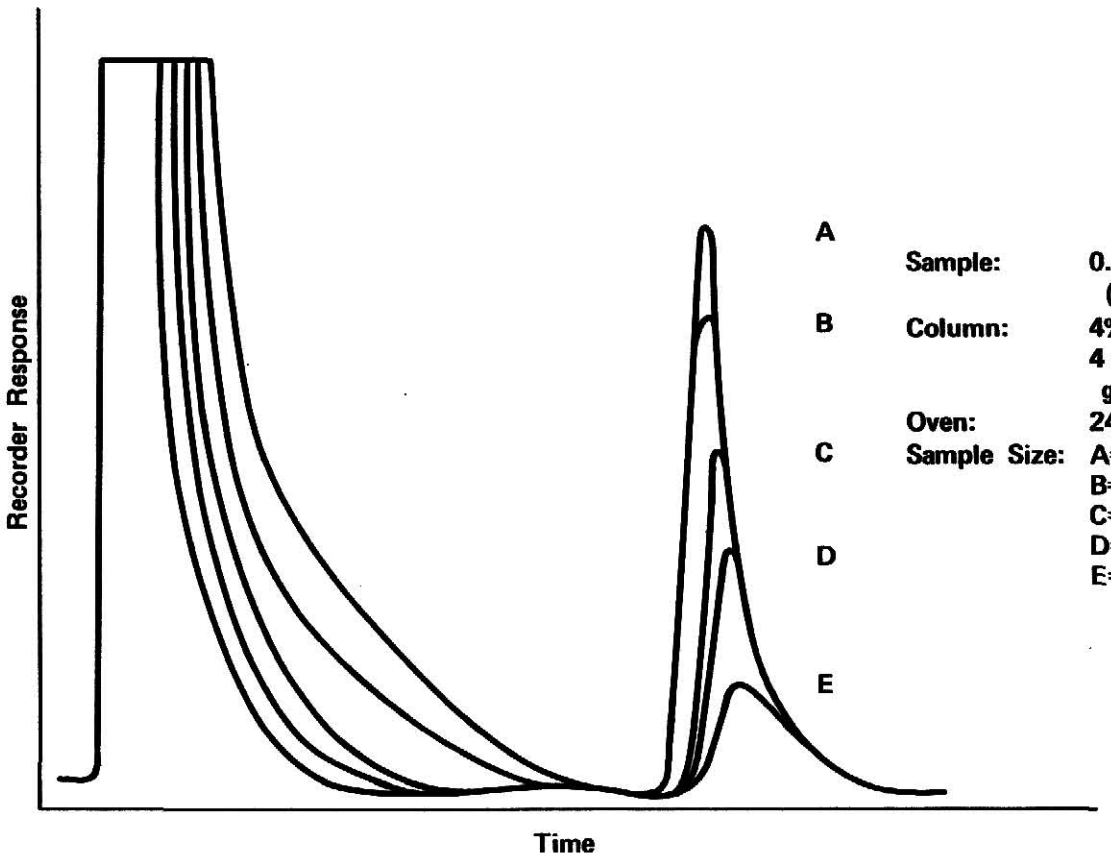
To rule out the possibility of a very fast irreversible reaction giving rise to this single elution peak, this peak was split off and collected. Enough material was collected to obtain a nuclear magnetic resonance spectrum and a mass spectrum of the trapped material. For comparison cholesterol samples were chromatographed under identical conditions on a column that had been acid washed and DMCS treated. A nuclear magnetic resonance spectrum of this trapped material was obtained. Also for comparison a mass spectrum of the commercial cholesterol sample from which the injected solutions were prepared was taken.

Two important peaks in the nuclear magnetic resonance spectrum indicate whether or not the cholesterol molecule has remained unchanged in the region of the molecule containing the -OH group, Fig. 10. The proton resonance peak at 6.5τ(TMS at 10.0τ) corresponds to the

EXPLANATION OF PLATE VI

Fig. 9. Chromatograms for a series of cholesterol injections of varying sample size.

Plate VI



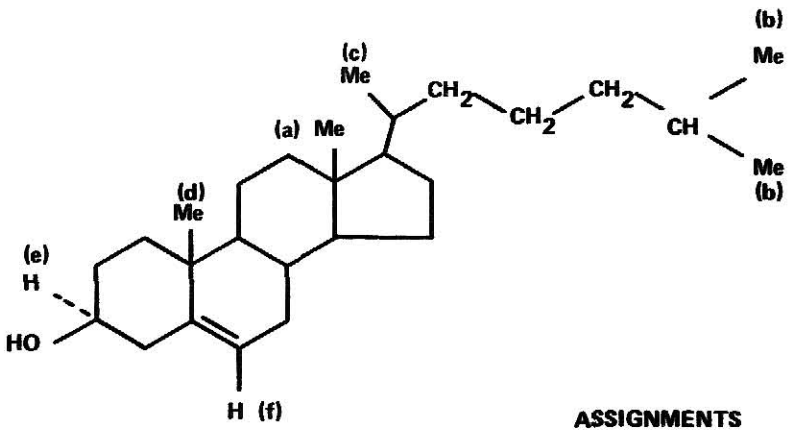
Sample:	0.1% cholesterol/benzene (varying size)
Column:	4% SE-30, naw Chromosorb W 4 mm x 3.5 ft. DMCS treated glass
Oven:	240°C
Sample Size:	A=2.9 μ 1 B=2.5 μ 1 C=2.0 μ 1 D=1.4 μ 1 E=1.0 μ 1

EXPLANATION OF PLATE VII

Fig. 10. Standard nuclear magnetic resonance spectrum of cholesterol. Spectrum #363, High Resolution NMR Spectra Catalog, Varian Associates, Palo Alto, California, 1962.

Plate VII

Cholesterol



ASSIGNMENTS

a 0.67	e 3.50
b 0.87	f 5.36
c 0.90	
d 1.00	

proton attached to the carbon atom at the 3 position, the carbon atom to which the -OH group is also attached. The second important resonance peak is at 4.64τ(5.36 ppm) and corresponds to the proton attached to the carbon atom on the double bond at the 6 position.

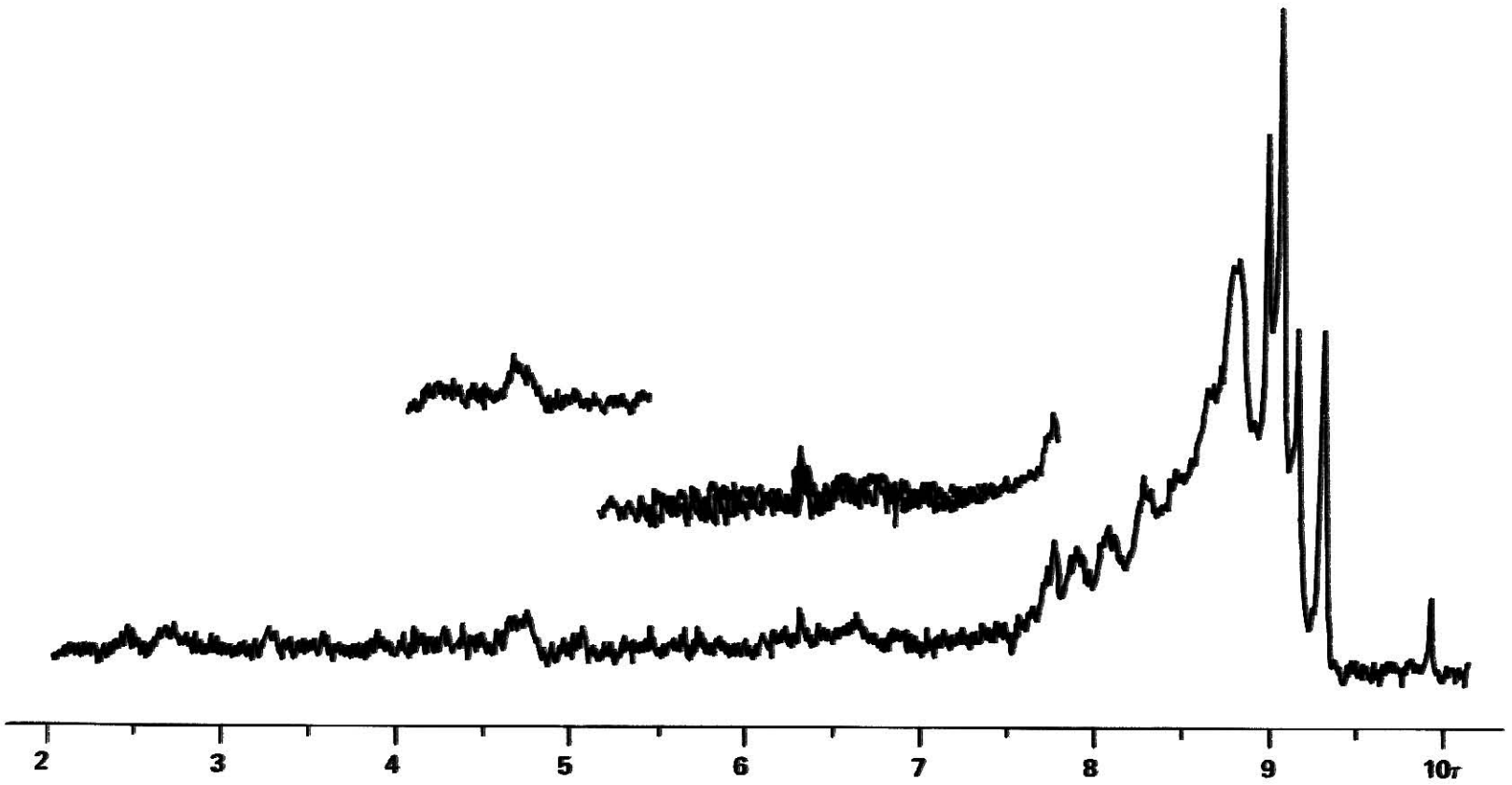
All three nuclear magnetic resonance spectra were essentially identical, Figs. 11,12,13. The two resonance peaks at 4.64τ and 6.50τ are clearly evident in the two spectra of the trapped material. These results indicate that no reaction of cholesterol in the region of the molecule containing the -OH group had taken place during chromatography on either the treated or the untreated column. The remaining portions of the spectra also match well.

The spectrum of the commercial cholesterol sample, Fig. 13, contained an impurity peak at approximately 6.88τ; this peak is not observed in either of the spectra of the cholesterol samples trapped upon elution from the chromatographic columns. The nuclear magnetic resonance spectra of both trapped cholesterol samples show a very small impurity peak at approximately 6.3τ, Figs. 11,12. The area of this peak is considerably less than the area of either of the peaks at 6.50τ and 4.64τ which result from the resonance of a single proton. The spectrum in Fig. 12 shows peaks at 2.78τ and 7.92τ for benzene and acetone respectively; these solvents were used to wash the trapped materials from the collection trap.

EXPLANATION OF PLATE VIII

Fig. 11. Nuclear magnetic resonance spectrum of cholesterol eluted from an untreated column.

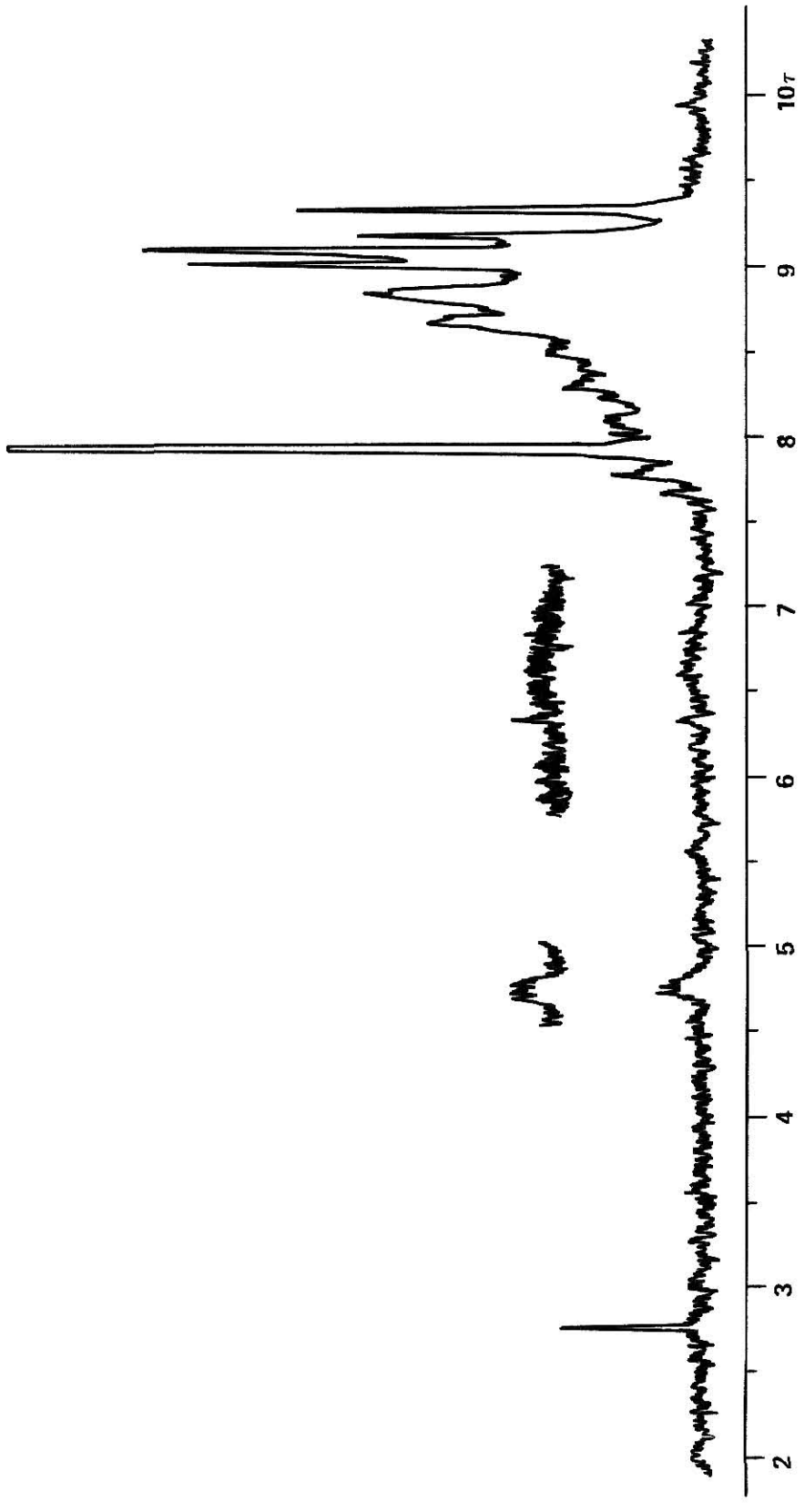
Plate VIII



EXPLANATION OF PLATE IX

Fig. 12. Nuclear magnetic resonance spectrum of cholesterol eluted from a treated column.

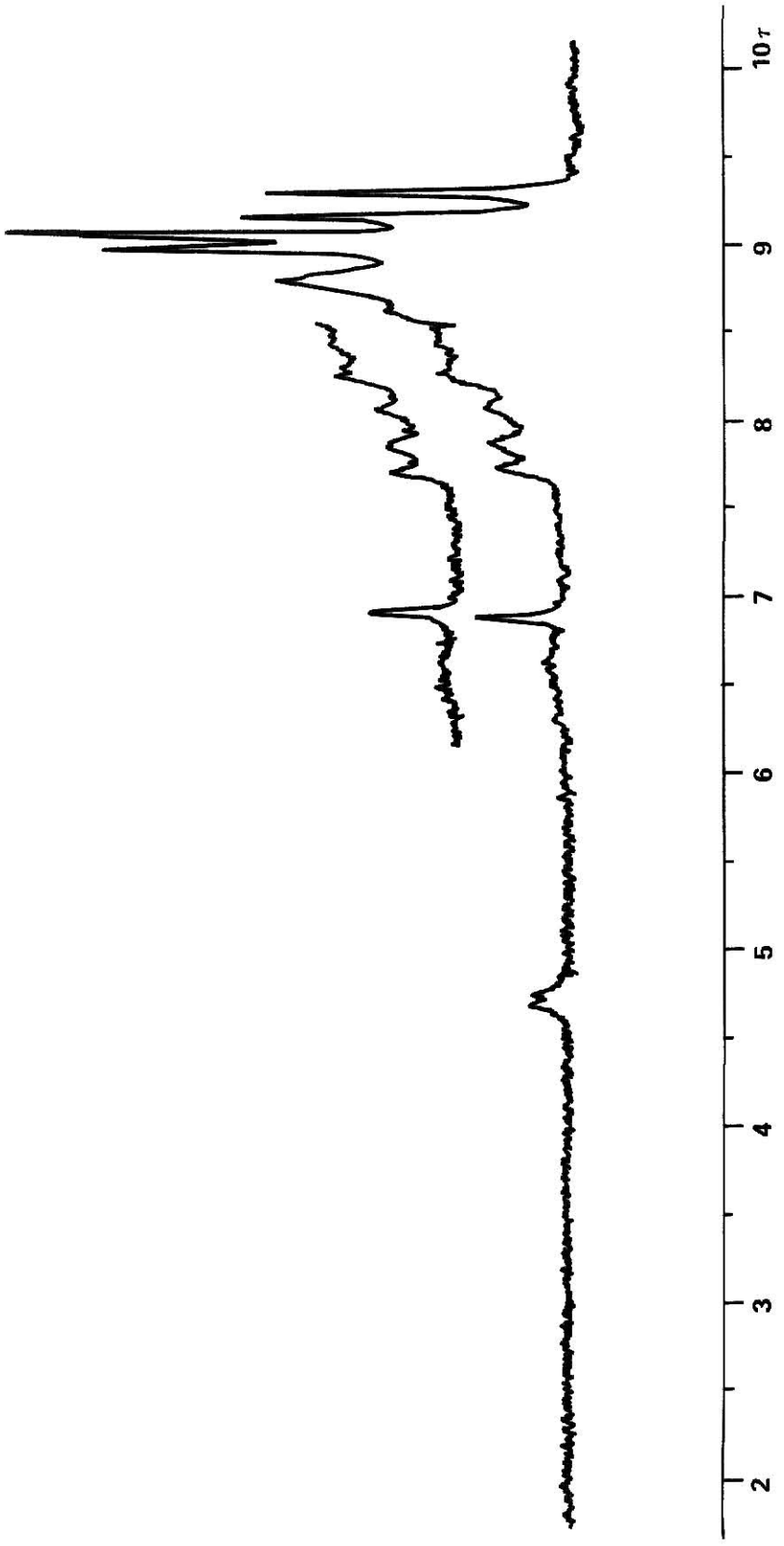
Plate IX



EXPLANATION OF PLATE X

Fig. 13. Nuclear magnetic resonance spectrum of commercial cholesterol sample.

Plate X



A mass spectrum of the trapped sample eluted from the untreated column was taken and compared with the mass spectrum of the commercial cholesterol sample. These two spectra are essentially identical, the parent ion at 386 mass units is clearly observed in both spectra. The spectrum of the eluted sample shows a very small parent ion +14 peak and an even smaller parent ion +16 peak. This peak at 386 +14 may arise by loss of H and gain of CH_3 by the cholesterol molecule during chromatography or storage in the glass trap via a reaction with the silylated glass surface; the amount of exchange appears to be quite small.

From this portion of the study it is concluded that contrary to the report of Spencer (58) the cholesterol molecule does not undergo a dehydration reaction during chromatography to form cholestadiene at the stated conditions. For smaller sample sizes the cholesterol samples give elution peaks showing significant tailing that results from non-linear adsorption. This tailing condition might well be expected for the chromatography of this molecule on a relatively low loaded column packed with an untreated diatomite support.

In an attempt to obtain an on-column reaction the column oven temperature was raised in 20°C increments, samples were injected, and chromatograms obtained. No indication of an on-column chemical reaction of cholesterol could be obtained below 270°C. At temperatures above 270°C

reactions involving cholesterol were definitely apparent; the cholesterol elution peak and three other major elution peaks were observed. Of these three product peaks, two are eluted before cholesterol and one after cholesterol. In addition two minor peaks were frequently observed. The first minor peak elutes between the solvent peak and the first product peak, lying very close to the first product peak. The second minor peak elutes between the second product peak and the cholesterol peak. By decreasing flow rate and by stopping the carrier gas flow for various time increments, the cholesterol peak may be observed to decrease in area while the three other major peaks in the chromatogram increase in area with increasing time spent on the column, Figs. 14,15, 16,17.

From the early stages of this study it became increasingly evident that the diatomite support plays a very important role in the observed phenomena. The first several injections of cholesterol onto a new, conditioned column always seemed to show a high reactivity. That is, the reactivity of the column appeared to be gradually reduced with each successive cholesterol injection, indicating that the reactive sites were gradually being destroyed or covered by the cholesterol samples. To show that the active sites were not destroyed by the cholesterol decomposition but simply blocked by "irreversible" adsorption of sample, a column with reduced reactivity was allowed to remain

EXPLANATION OF PLATE XI

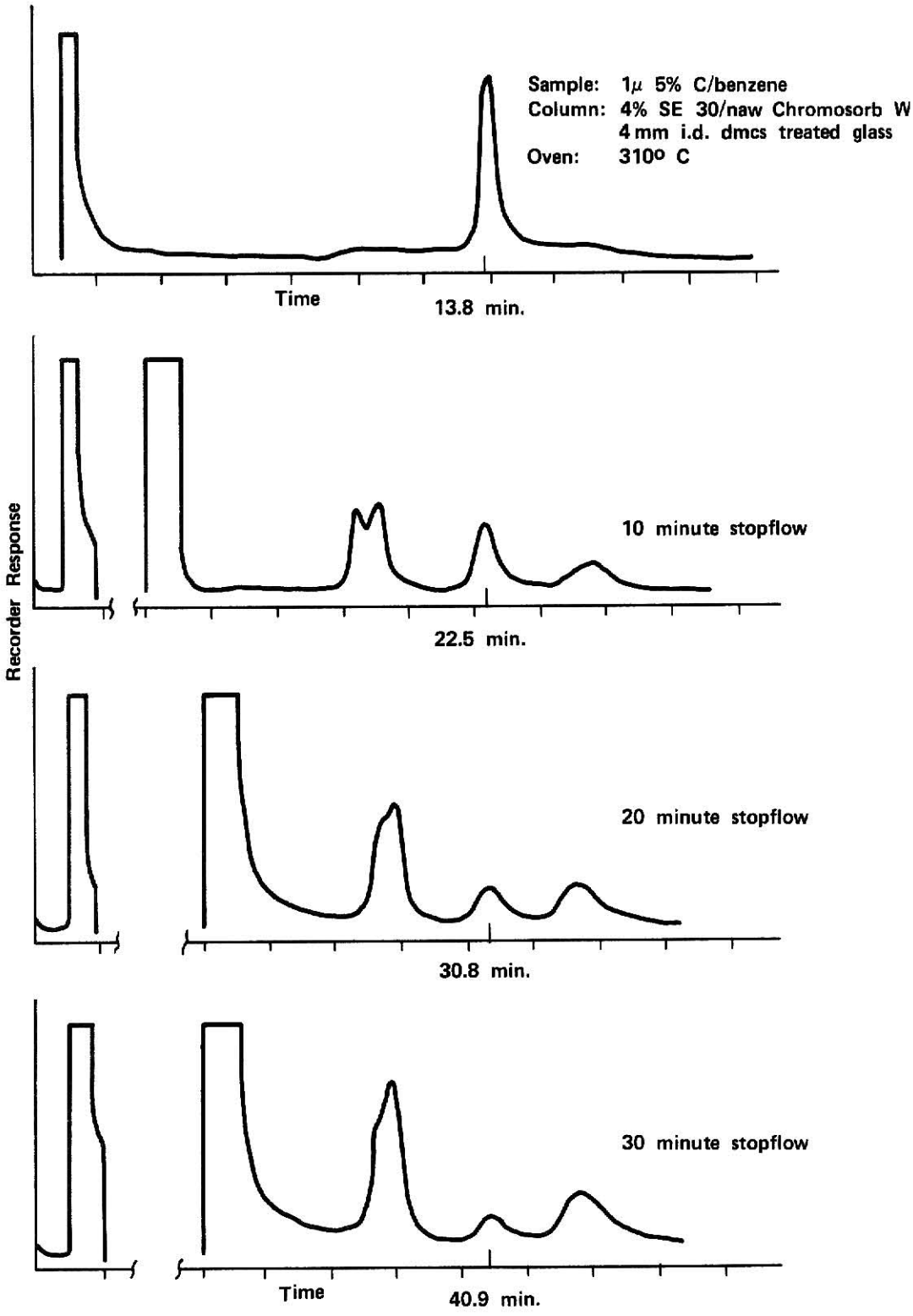
Fig. 14. Chromatogram of on-column cholesterol reaction.

Fig. 15. Chromatogram of on-column cholesterol reaction. 10 minute stop-flow.

Fig. 16. Chromatogram of on-column cholesterol reaction. 20 minute stop-flow.

Fig. 17. Chromatogram of on-column cholesterol reaction. 30 minute stop-flow.

Plate XI


Sample: 1μ 5% C/benzene
Column: 4% SE 30/naw Chromosorb W
4 mm i.d. dmcs treated glass
Oven: 310° C
Time
13.8 min.
Recorder Response
10 minute stopflow
22.5 min.
20 minute stopflow
30.8 min.
30 minute stopflow
Time
40.9 min.

overnight at 290°C with a mobile phase flow. The first several injections of cholesterol showed significantly higher reactivity than the last injections made the night before. Because the retention volume of the cholesterol peak was not significantly changed, it is believed that this increase in column reactivity can not be attributed totally to stationary phase bleed uncovering new sites, but must involve bleed of adsorbed cholesterol sample. It is felt that these general observations are in accordance with the above mentioned practice of saturating a column with compounds that display strong tailing characteristics prior to injection of the sample to be analyzed. These observations also suggested the technique of packing a pre-column of uncoated and untreated diatomite support to increase product yield for trapping and observing the reaction.

In addition to loss of column reactivity with numerous sample injections one other observation was made. As the reactivity of the column decreased, a definite loss of separation of the first two product peaks was observed. With each successive sample injection the separation of these two peaks became poorer and poorer until only a single peak was observed. This observation seemed to indicate that perhaps an adsorption process involving the active sites was responsible for this separation rather than a partition process with the SE-30 stationary phase.

To test this hypothesis, a 3 foot DMCS treated glass column packed only with uncoated, untreated Chromosorb W was placed in the column oven. With chromatographic conditions the same as in the previous experiments (column oven 290°C etc.) samples of cholesterol in benzene were injected. Chromatograms containing two distinct elution peaks on the tail of the benzene solvent peak were observed, Fig. 18. For comparison a sample of eicosane in benzene was injected, giving a chromatogram with a single sharp elution peak on the tail of the benzene solvent peak.

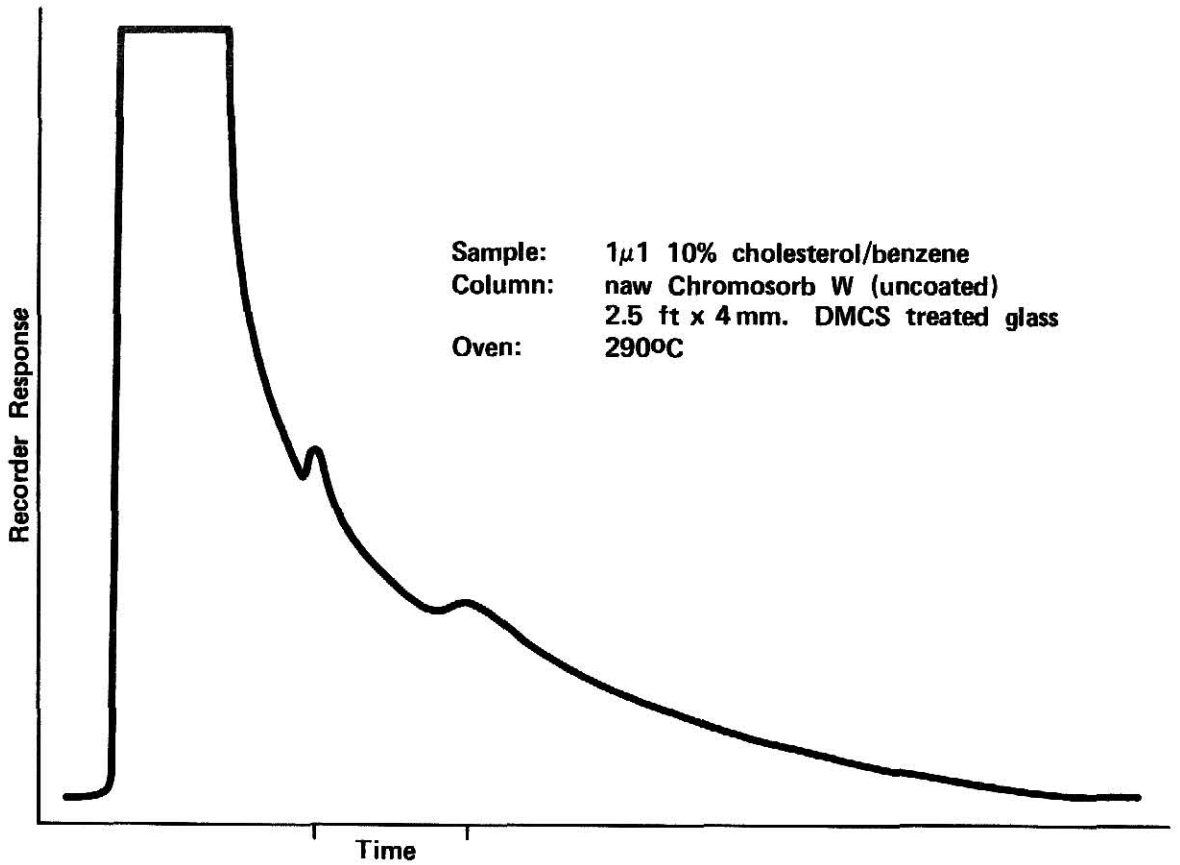
An attempt was made to determine if the silanol groups or the mineral impurities on the surface, are responsible for the diatomite reactivity with cholesterol. A column packed with 3% SE-30 on acid-washed Chromosorb W with a pre-column of acid-washed Chromosorb W was placed in the column oven at 290°C. This column displayed as nearly the same reactive characteristics as the non-acid-washed columns as could be determined.

After several samples had been passed through this column and the observations made, the column temperature was reduced to below 150°C and three, 5µl samples of Silyl 8 were injected. The column was allowed to flush with the normal carrier gas flow for two hours. Silyl 8 is a commercial material designed for in-situ treatment of the chromatographic support. The column temperature was then

EXPLANATION OF PLATE XII

Fig. 18. Chromatogram of cholesterol injection onto a column of untreated and uncoated Chromosorb W.

Plate XII


Recorder Response
Time
Sample: 1μ1 10% cholesterol/benzene
Column: naw Chromosorb W (uncoated)
2.5 ft x 4 mm. DMCS treated glass
Oven: 290°C

returned to 290°C and several cholesterol samples were injected. The reactivity of the column had been significantly reduced by the Silyl 8 treatment. A small amount of reaction could, however, still be observed. The Silyl 8 treatment was then repeated. When the column had been returned to 290°C, a cholesterol sample gave a chromatogram that still displayed a very small amount of reaction. Samples were then chromatographed at 300°C and 310°C, giving similar results. Although the support could not be completely deactivated, cholesterol samples could be satisfactorily chromatographed at 300°C without significant reaction after in-situ column treatment with Silyl 8. These results lead to the conclusion that it is primarily the silanol groups on the support surface that catalyze the cholesterol reaction.

Enough material from each product elution peak was split off and collected to allow rechromatography of the products and the obtaining of a mass spectrum of each component. Because of the small sample size and relatively long elution times required to obtain significant product yield, collecting enough material for analysis was a serious problem. Injection of larger samples gave rise to momentary crystallization of the sample in the injection port; this condition results in a very seriously fronting cholesterol elution peak. The most practical solutions to the problems of obtaining significant yield of the products while maintaining adequate separation

and a relatively short elution time were to use a packed pre-column of non-acid-washed Chromosorb W and a stop-flow technique. The cholesterol sample was injected, a few seconds were allowed for the sample to reach the pre-column, and then the carrier gas flow was stopped, allowing the sample to remain on the untreated pre-column for a given time. The carrier gas flow was then returned to normal and the products and reactant eluted off the column.

None of the product peaks trapped was found to be cholestadiene; in fact, none of the peaks was found to have a molecular weight less than that of cholesterol, ruling out the possibility of a simple dehydration reaction.

The first peak to elute has a probable molecular weight of 434 mass units. In addition, the mass spectrum of this material shows major peaks at 361,316, and 290 mass units.

The second product peak to elute has a probable molecular weight of 534 mass units and shows major peaks in the mass spectrum at 506, 451, 429, and 400 mass units.

The mass spectrum of the third product peak has major peaks at 413, and 399 mass units.

In none of the mass spectra of the three products could the loss of an -OH fragment be observed. It is,

therefore, believed that none of these products contains an -OH group; or, if one does contain an -OH group, this group is at a very protected position in the molecule.

There are two arguments that prove that the high mass peaks seen in the mass spectra of these products do not arise from components of normal bleed or thermal decomposition of the stationary phase. First, the mass spectrum of the cholesterol peak trapped under identical conditions compared well with the mass spectrum of the commercial sample that was used to prepare the solutions for injection. The mass spectrum of the trapped cholesterol material showed only two very small peaks at parent ion +14 and parent ion +16 that were not observed in the mass spectrum of the commercial sample.

Second, communications with Robinson and Coutant (55) indicate that none of the major mass peaks observed in the mass spectra of components of stationary phase bleed or thermal decomposition under chromatographic conditions of dimethylsilicone ploymers similar to SE-30 could be observed in any of the mass spectra of these three product elution peaks. Mass spectra of column bleed and thermal decomposition components of dimethylsilicone ploymers show characteristic peaks at m/e = 73 for the $(CH_3)_3$-Si-group, m/e = 207 for $Si_3(CH_3)_5O_3$(usually the largest peak for thermal decomposition components), m/e = 221 for $Si_3(CH_3)_7O_2$, m/e = 281 for $Si_4(CH_3)_7O_4$, and m/e = 295 for $Si_4(CH_3)_9O_3$.

Because not one of these peaks could be observed in any of the mass spectra of the trapped product peaks, it is believed, that the high mass peaks observed in the mass spectra are not artifacts of stationary phase bleed or decomposition, but, arise from products formed in the cholesterol reaction. With molecular weights above 400 identification of the structure of these products would be particularly difficult.

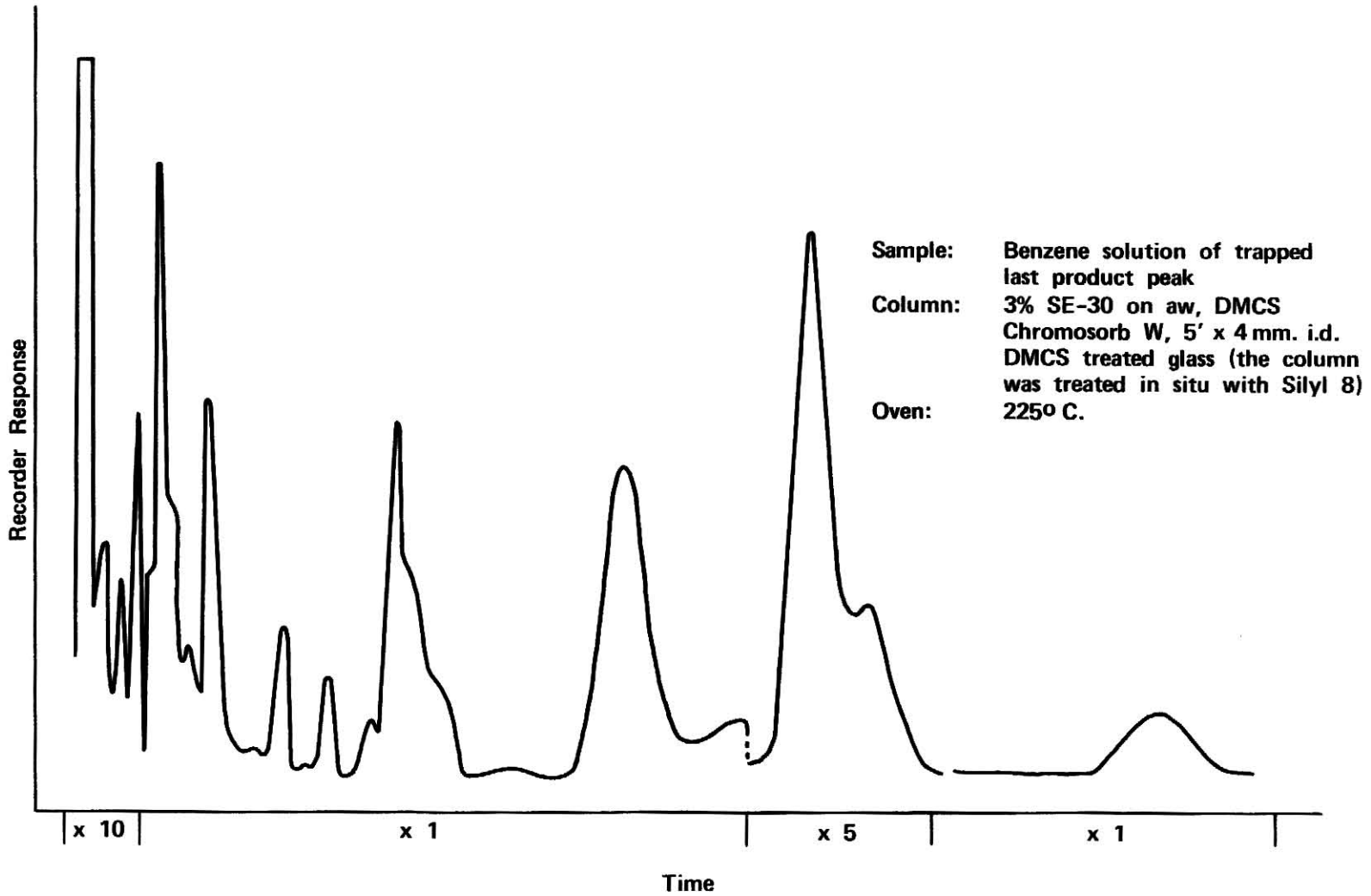
Rechromatography of the trapped product peaks proved an early suspicion. Frequently, chromatography of cholesterol samples gave chromatograms showing only what was thought to be the first two product peaks, the cholesterol peak and the third product peak not being observed. After a series of sample injections had decreased the column's reactivity these two peaks were again observed. That this third product peak was not always observed seemed to indicate that this product may be a precursor of one or both of the first two products to elute or may simply be decomposing to give a component or components that are not being observed. Rechromatography of the trapped material from the third product peak on a non-reactive column (acid-washed and DMCS treated) gave a chromatogram shown in Fig. 19.

From this chromatogram it is evident that this material has undergone further decomposition giving the numerous elution peaks that appear on the tail of the solvent peak and throughout the chromatogram. Because this product

EXPLANATION OF PLATE XIII

Fig. 19. Chromatogram of trapped material of the last product peak to elute. Rechromatographed on a deactivated column.

Plate XIII


Recorder Response
Sample: Benzene solution of trapped last product peak
Column: 3% SE-30 on aw, DMCS Chromosorb W, 5' x 4 mm. i.d. DMCS treated glass (the column was treated in situ with Silyl 8)
Oven: 225° C.
x 10
x 1
x 5
x 1
Time

peak could not be totally separated from the tailing cholesterol peak during trapping, the cholesterol peak and its decomposition products appear in this chromatogram; therefore, it is not possible to prove that this product of the cholesterol reaction generates one or both of the other two main product peaks.

Rechromatography of the first two product peaks gave chromatograms showing a single elution peak in each case; however, retention measurements for these two peaks were not readily comparable with retention measurements for these peaks when generated during an on-column cholesterol reaction. That these materials elute before cholesterol on an SE-30 column but show by mass spectroscopy molecular weights greater than cholesterol may indicate that these two materials also are not chemically stable to the trapping, storage, and rechromatography conditions used in this study.

With at least three product peaks present and considerable peak tailing attributed to adsorption phenomena, the models developed by van Swaay could not be readily applied. Considerable effort was, however, directed towards obtaining kinetic information using peak area measurements. Although general trends could be observed, reliable measurements of kinetic parameters could not be made. Results could not be accurately reproduced and the temperature dependence of the reaction could not be measured with any degree of certainty. To make these calculations based on peak area measurements,

the assumption of fast linear adsorption of reactant (cholesterol) independent of the presence of products must be made. From the above study a fast linear adsorption of cholesterol is obviously not a valid assumption; some cholesterol is lost to "irreversible" adsorption.

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THE REACTION OF CHOLESTEROL WITH UNTREATED CHROMOSORB W DURING GAS CHROMATOGRAPHIC ELUTION

by

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AN ABSTRACT OF A MASTER'S THESIS

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The interaction of sensitive steroid molecules with the diatomaceous earth support material during gas chromatographic elution prevented the rapid development of gas chromatographic steroid analysis until adequate support deactivation techniques had been developed. Peak tailing, irreversible loss of sample, and chemical reaction of the sample have been frequently reported. In most situations these observations, which are characteristic of solute support interactions, have been viewed as the introduction of an intolerable uncertainty into the analytical results; in some situations, however, useful information may be obtained about the conditions that contribute to peak asymmetry. The initial effort of this study was an attempt to apply the models developed by van Swaay (Advances in Chromatography, Vol. 8 (J. C. Giddings and R. A. Keller, Eds.), Dekker, New York, 1969, p 363.) for the extraction of reaction kinetic parameters from the distortion of a gas chromatographic elution peak to the dehydration of cholesterol to cholestadiene reported by Spencer (Instrumentation in Gas Chromatography (J. Krugers, Ed.), Centrex Publishing Company, Eindhoven, 1968, p 131.)

The dehyration of cholesterol to cholestadiene at the conditions reported by Spencer could not be reproduced. Chromatograms showing a single tailing elution peak were obtained. Although these chromatograms appeared to be similar to the one given by Spencer, nuclear magnetic resonance spectroscopy and mass spectroscopy of trapped material from this peak proved the peak to be unreacted cholesterol. Injection of a series of sample of various sizes

indicated that the observed tailing results from non-linear adsorption and not chemical reaction during chromatography.

At column oven temperatures above 270°C a cholesterol decomposition catalyzed by untreated diatomaceous earth support was observed. A cholesterol peak and at least three product peaks could be observed. Using various flow rates and stop-flow techniques, attempts were made to obtain kinetic parameters from cholesterol peak area measurements; however, reproducible results could not be obtained because of irreversible loss of some sample during chromatography.

Rechromatography of material trapped from the two product peaks eluting before cholesterol on an SE-30 column gave single elution peaks. Because mass spectroscopy of these materials indicated that both had molecular weights higher than cholesterol, the stability of these two products to the trapping techniques is questionable. Rechromatography of trapped material from the third product peak gave a chromatogram showing numerous components, indicating that this product was definitely not stable to the trapping, storage, and rechromatography conditions used.

Experiments in this study indicate that the cholesterol interactions with the support surface that give rise to this decomposition take place at Si-OH sites. These sites may be deactivated using conventional techniques of silylation.