Residual Ethylene Oxide and Ethylene Glycol in Ethylene Oxide Sterilized Pharmaceuticals

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The development and application of two methods for the determination of sterilization residues in ethylene oxide sterilized steroids, vitamins, and antibiotics are described. One method is based on the separation and concentration of ethylene oxide (ETO) by distillation and measurement by internal standard gas liquid chromatography. The other method, based ultimately on the colorimetric determination of formaldehyde, permits determination of total glycol as well as specific ETO and glycol after ion exchange or extraction separation procedures. The mechanism of retention is discussed, with particular emphasis on solvation phenomena.

THE POSSIBLE presence of residues resulting from the ethylene oxide (ETO) sterilization of heat-labile pharmaceutical products is of scientific and legal concern. However, although the sterilizing effect of ETO on food and pharmaceuticals has been studied extensively (1–6), little attention appears to have been given to the quantitative determination of sterilization residues and to the mechanism of their retention. Published work has been restricted to ETO residues (7) and has shown that ETO is present in fumigated spices in the parts per million range. The levels of decomposition products of ETO have not been considered, although the presence of ethylene glycol (glycol) in sterilized pine shavings has been demonstrated qualitatively by chemical and physiological means (8). In this study, therefore, quantitative methods for both ETO and glycol were developed and applied to experimental batches of selected pharmaceuticals, including steroids, antibiotics, and vitamins. The results are interpreted in terms of possible mechanisms of retention.

Many of the methods proposed for the analysis of ETO are unsuitable for trace levels. The only reported direct colorimetric method for ETO, based on the reaction of lepidine with volatile epoxides (9), although sensitive, requires rigorous control of reaction conditions. Indirect colorimetric procedures, based ultimately on the determination of formaldehyde after hydrolysis of ETO to glycol and periodate oxidation of the glycol, have employed phenylhydrazine (7) and chromotropic acid (7, 10) as reagents. Other spectrophotometric as well as spectrophotofluorimetric procedures (11, 12) presumably could be used also. However, none of the above methods offer the sensitivity or specificity of gas liquid chromatography (GLC).

In this work, ETO is isolated and concentrated by distillation and determined by GLC. Glycol and total glycol (glycol plus ETO as glycol) are determined by a modification of the chromotropic acid reaction, after separation of the glycol from interfering substances by extraction or by ion-exchange chromatography.

ETHYLENE OXIDE

Experimental

Reagents.—Propylene oxide (PrO) and ETO were obtained from Matheson, Coleman and Bell, East Rutherford, N. J. Dioxane was purified when necessary by fractional crystallization. Acetone was purified by distillation.

Sterilization.—Thin layers of the samples to be sterilized were exposed to a gas mixture containing about 10% ETO and 90% CO_2[1] in commercial and laboratory scale equipment at 18 p.s.i. for 24–72 hr. at room temperature and subsequently were subjected to vacuum purging for at least 2 hr. The treated samples were stored in septum-sealed vials for at least 1 month prior to assay.

Distillation.—A weighed sample, usually about 1–5 Gm., was added to a chilled 250-ml. round-bottom flask containing several boiling chips. About 50 ml. of chilled dry solvent was added, and the flask then was connected immediately to the still. For the recovery experiments, ETO in dilute acetone solution was added quantitatively to the previously distilled and rechilled charge *via* the Dumas bulb technique.

Distillations were performed with a jointless PCVR still (13) fitted with a tared silicone septum-sealed receiver vial (14). About 300 mg. of acetone was added to the receiver at the start of the distillation. The vent line was protected with anhydrous calcium sulfate, and the receiver was immersed in dry ice. Distillations were performed at a rate of <1 ml./hr. Upon completion, the receiver was brought to room temperature in a desiccator and the net weight of its contents determined. Distillates were assayed as soon as possible (usually within several hours) after collection.

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[1] Marketed as Carboxide by Union Carbide Corp.

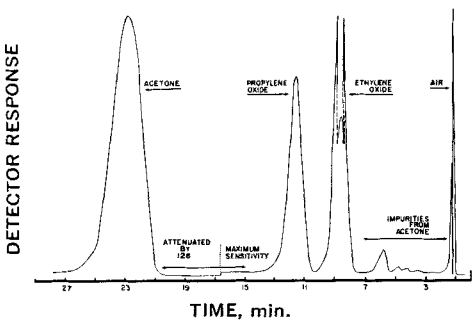

Fig. 1.—Typical gas liquid chromatogram.

GLC Assay.—A Perkin-Elmer model 154-B Vapor Fractometer with thermal conductivity detector was used for the GLC assays. Helium was used as the carrier gas at a flow rate of 45 ml./min. Most of the work was done on a 2-M. 0.25 -in. o.d. copper column containing 25% dimethyl sulfolane on 30–60 mesh firebrick at 30°. The work was shifted to a column consisting of 1 M. of 25% 1,2,4-butanetriol in series with 2 M. of 25% polyethylene glycol 1500 operated at 40°, however, when it was discovered that trace impurities in the solvents, which previously had to be removed, were now eluted at a noninterfering retention time. The columns were first conditioned at 80–90°, then further conditioned by injecting large samples of ETO in acetone.

The septum seal weighing and transfer technique (14) was used extensively. Hard silicone rubber septums (The West Co., Phoenixville, Pa.) were used for their elasticity at low temperatures. Microliter syringes and needles were chilled and dried before making transfers.

The assays were performed by the internal standard technique using propylene oxide (PrO) as the standard. Calibration was effected with 20 μl. of a 0.2 wt. % solution in acetone of a 1.5:1 weight mixture of PrO–ETO. The calibration mixture was prepared in bulk and subdivided in 1-ml. quantities in flame-sealed ampuls. Fresh ampuls were used daily, after transferring their contents to septum-sealed vials. A prerun of all sample distillates was made, both to insure the absence of components with a retention time similar to that of the internal standard and to allow estimation of the amount of PrO to be added to an aliquot of the distillate to bring the ratio of PrO to ETO into the calibrated relative concentration range.

Results

Selection of Solvent.—To determine accurately the residual ETO content of solid samples, it was apparent at the outset that an initial step involving complete solution of the sample was necessary. Although many individual solvents were considered, none was found to possess all of the properties required for its subsequent use in the isolation and concentration of ETO by distillation and the assay of ETO by GLC. A two-component solvent was therefore devised. Acetone (b.p. 56.5°), 10%, was used to moderate the distillation temperature, to sweep the ETO from the still, and to serve as a relatively rapidly eluted chromatographic carrier solvent. Higher boiling pyridine (b.p. 113.5°), 90%, was used for its solvent powers. However, part of the way through the investigation it was found that pyridine led to reaction errors, and it was replaced by dioxane (b.p. 101°) for subsequent work with the steroids. Benzyl alcohol (b.p. 205°), 90%, proved satisfactory for the penicillin-streptomycin formulation.

Separation and Concentration of ETO.—Previous workers have used gas washing techniques to strip ETO from a solution of the substance under assay. Gunther *et al.* (9) used nitrogen to sweep a sodium isopropoxide solution, with 94–96% recovery of 2–90 mcg. Critchfield and Johnson (7), using air to sweep the ETO from boiling water solutions of spices, reported a maximum recovery of 86%. Careful calibration of conditions is required in the latter case, as the rate of hydrolysis of ETO depends on concentration, pH, and solute effects as well as on time and temperature.

Table I.—Precision of the Gas Liquid Chromatographic Assay for Ethylene Oxide

Soln.	Runs, No.	Wt./Ratio, PrO/ETO	R^a	±Av.D.	K^b	±Av.D.
1[c]	4	0.884	0.578	0.004	1.58	0.03
2	1	1.363	0.583	...	1.59	...
3	1	1.413	0.580	...	1.60	...
4	2	2.571	0.581	0.001	1.58	0.01

[a] R is the ratio of the corrected retention times of ETO to PrO on the dimethyl sulfolane column. [b] K is the calculated calibration constant (peak height ratio, ETO/PrO, times weight ratio, PrO/ETO). [c] Runs made on 3 successive days.

Table II.—Recovery of Ethylene Oxide from Steroid and Vitamin Mixture Solutions

Substance	Water,[a] mg.	Ethylene Oxide Added, mg.	Ethylene Oxide Recovered, %
Solvent blank	...	0	0
Solvent[b]	...	1.7	95
Cortisone acetate wet cake	3000	4.8	71
Hydrocortisone acetate	25	3.6	89
Hydrocortisone *tert*-butyl acetate	87	5.4	93
Hydrocortisone *tert*-butyl acetate	...	22.3	92
Prednisolone *tert*-butyl acetate	100	3.1	93
Vitamin mixture[c]	...	24.5	96

[a] Water content of the 5-Gm. sample used for the assay. [b] The solvent was 90% pyridine, 10% acetone. [c] Contains thiamine hydrochloride, riboflavin, niacinamide, calcium pantothenate, pyridoxine hydrochloride, ascorbic acid, and cyanocobalamin, among other components.

TABLE III.—DEMONSTRATION OF LOW RECOVERIES OF ETHYLENE OXIDE IN THE PRESENCE OF THE PYRIDINE–WATER SYSTEM

Distillation Soln.	Age[a] before Distillation, hr.	Ethylene Oxide Added, mg.	Ethylene Oxide Recovered, %
80% Pyridine, 20% acetone	24	54.8	94
80% Pyridine, 20% acetone, plus 2% H_2O	0	50.9	87
80% Pyridine, 20% acetone, plus 2% H_2O	24	15.0	80
80% Dioxane, 20% acetone	0	9.6	95
80% Dioxane, 20% acetone, plus 2% H_2O	0	34.1	96
80% Dioxane, 20% acetone, plus 2% H_2O	24	17.0	94

[a] Stored at −5°C.

TABLE IV.—GAS CHROMATOGRAPHIC IDENTIFICATION OF ETHYLENE OXIDE IN THE DISTILLATE FROM PREDNISOLONE *tert*-BUTYL ACETATE

Chromatographic Col. Substrate[a]	Length, M.	R, ETO/PrO Known Mixture	R, ETO/PrO Sample
Di-*n*-decyl phthalate	2	0.474	0.474
Dimethyl sulfolane	2	0.575	0.571
Polyethylene glycol 200	2	0.680	0.678
Polyethylene glycol 200 + 1,2,4-butanetriol	2 + 1	0.735	0.734
Polyethylene glycol 1500 + 1,2,4-butanetriol	2 + 1	0.701	0.703

[a] All substrates were on 30–60 mesh firebrick in 0.25-in. o.d. copper columns.

In this work, ETO is both separated from the sample solution and concentrated by slow fractional distillation at a high reflux ratio in an efficient still (13). Although varying geometries of receiver containers were used (14), typical conditions called for the addition of about 300 mg. of acetone to the receiver to act as a vapor trap and distillation of a 500 mg. fraction. A second distillation fraction of 200–300 mg. was collected separately; in every case, testing by GLC indicated the absence of ETO in this fraction.

GLC Assay.—A typical chromatogram is shown in Fig. 1. The precision of the GLC assay was checked by replicate runs on replicate calibration solutions. Peak heights were used, since they gave results that were equivalent to those from peak areas. The results are shown in Table I. The precision of R, the ratio of corrected retention times of ETO to PrO, demonstrates the utility of this ratio for qualitative identification. The values of K, the calibration constant (peak height ratio, ETO/PrO, times weight ratio, PrO/ETO), indicate that the precision of the quantitative aspects is within 2%. The data also show that K does not depend on the relative weights of ETO and PrO over at least a threefold relative weight range. Calibration with respect to absolute concentration indicated linearity up to an injection of at least 0.5 mg. of ETO.

Sensitivity.—The sensitivity of the method is limited primarily by the sample size, since the method contains a concentration step. As an order of magnitude, however, if the volume of the distillate of a 1-Gm. sample is 1 ml. (after addition of internal standard) and a 50-μl. aliquot is injected, the sensitivity of detection is better than 10 p.p.m. ETO in the sample under the given experimental conditions. In practice, the use of larger samples, concentration to smaller volumes, and occasionally, the injection of larger aliquots (up to 100 μl. could be tolerated), gave sensitivities down to 0.1 p.p.m. with a thermal conductivity detector. More sensitive detectors, not available at the time this work was performed (1959–1960), would obviously permit greater sensitivity.

Recovery and Accuracy.—The over-all accuracy of the method was determined by conducting recovery experiments in the presence of at least one lot of each type of sample. This was done by adding a known amount (2–25 mg.) of ETO to the sample stillpot residue after that solution had first been distilled and redistilling. The results are listed in Table II. Omitting the cortisone acetate

TABLE V.—RESIDUAL ETHYLENE OXIDE CONTENT OF CARBOXIDE STERILIZED PHARMACEUTICALS

Sample	No.	Weight, Gm.	% ETO[a]
Cortisone acetate wet cake	*1*	5	<0.0001
Cortisone acetate wet cake	*2*	5	<0.0001
Hydrocortisone acetate	*1*	5	<0.0001
Hydrocortisone *tert*-butyl acetate	*1*	5	0.90
Hydrocortisone *tert*-butyl acetate	*2*	5	1.63
Hydrocortisone *tert*-butyl acetate	*2*	2	1.61
Hydrocortisone *tert*-butyl acetate	*2*[b]	2	0.51
Prednisone	*1*	2	0.0001
Prednisolone *tert*-butyl acetate	*1*[c]	5	<0.0001
Prednisolone *tert*-butyl acetate	*2*	5	0.80
Prednisolone *tert*-butyl acetate	*2*[b]	3	0.12
Vitamin mixture	*1*[d]	5	<0.0005
Vitamin mixture	*2*[d]	5	<0.0001
Antibiotic formulation	*1*[e]	5	<0.0005

[a] Values preceded by the symbol < represent a conservative limit of detection sensitivity for the particular run; no sign of ETO was observed in these runs. [b] Poststerilization vacuum treatment time was 8 hr.; vacuum treatment time for all other sterilized samples was 2 hr. [c] Control run, composite of unsterilized samples. [d] Samples have the same final composition (*cf.* Table II) but were prepared by different techniques. Number *1* was prepared by freeze drying an aqueous solution of the components, followed by milling; No. *2* is a physical mixture of the individual crystalline components. [e] Formulation contained streptomycin, penicillin and procaine, among other components.

result, the average recovery is 93% with a standard deviation of 1%.

The atypically low recovery for cortisone acetate, the sample with the highest water content, suggested that the additional loss of ETO occurred by chemical reaction rather than by wholly physical means. To investigate this possibility, both pyridine and neutral dioxane solvents were prepared for distillation with and without the addition of 2% water. The solutions were aged for the time indicated at −5° but otherwise were treated by the usual sample procedure. The results in Table III indicate that losses greater than 5% are due to the use of pyridine in the presence of water. The reactions responsible for the loss are undoubtably the base-catalyzed hydration of ETO (15) and the condensation of ETO and pyridine to form *N'*-(2-hydroxyethyl) pyridinium hydroxide (15, 16). Subsequent work was performed with neutral solvents (dioxane, benzyl alcohol) to eliminate this source of error.

Identification.—The identity and homogeneity of the material measured in the assay was confirmed as pure ETO by demonstrating that its chromatographic behavior, indicated by the retention time relative to PrO and by the symmetry of its band, was identical to that of known ETO on five different column systems under somewhat different conditions. The results are listed in Table IV.

Results.—Typical results are listed in Table V for the assay of various steroids, vitamins, and antibiotics for residual ETO.

RESIDUAL ETHYLENE GLYCOL

A modification of the well-known periodate oxidation and chromotropic acid color reaction for formaldehyde (7, 10, 17) was selected for the determination of glycol. Preliminary separations were required to obtain the glycol in a state free of interfering substances. With appropriate variations, this approach permits determination of specific residual glycol (SRG), specific residual ETO (SRO), and total residual glycol (TRG, the sum of SRG and SRO). TRG is determined by assaying for glycol after the sample is submitted to conditions resulting in quantitative hydrolysis of ETO. SRG is determined after removing any residual ETO from the samples by sparging with nitrogen. In both cases, the blank consists of the similarly assayed unsterilized sample. The difference in glycol content between TRG and SRG, after correcting for the molecular weight difference between ETO and glycol, is then attributed to ETO. Alternatively, SRO may be determined more directly by a differential hydrolysis procedure that does not require an unsterilized sample blank. Here, the sample is assayed for glycol under the hydrolysis conditions before and after the removal of ETO from the sample.

Experimental

Reagents and Apparatus.—Chromotropic acid (CA; 4,5-dihydroxy-2,7-naphthalene disulfonic acid) was purified by recrystallization from a water–ethanol system after filtration through a Celite 545 filter bed.

Chloroform.—This was washed three times with water, dried with anhydrous sodium sulfate, and passed through a 1-in. i.d. column containing 5 in. of acid-washed alumina on top of 0.5 in. of silica gel. Only lots showing a negligible blank by the assay are used.

Periodic Acid, 0.1 N.—Dilute 7.0 Gm. of $HIO_4 \cdot 2H_2O$ and 3 ml. of concentrated H_2SO_4 to 250 ml. with distilled water.

Sodium Arsenite, 0.25 M.—Add 50 ml. of 3 *N* NaOH to 12.5 Gm. of As_2O_3. After complete solution is achieved, neutralize to a faint red (phenolphthalein indicator) with 1 *N* H_2SO_4. Dilute to 250 ml. with water.

CA, 1% in Concentrated H_2SO_4.—Prepare fresh immediately before use and run a blank with each assay.

Resins.—The analytical grade resins supplied by Bio-Rad Laboratories, Berkeley, Calif., were used. Anion exchange resin, AG-1-X8, 50–100 mesh, chloride form, was converted to the hydroxide form by passing 1 *N* NaOH through it until the eluate gave a negative test with $AgNO_3$. It then was washed with water until neutral, decanted several times to remove fines, and stored under water in an amber bottle. Cation exchange resin, AG-50W-X8, 50–100 mesh, hydrogen form, was converted to the sodium form with 1 *N* Na_2SO_4, washed with water, decanted several times to remove fines, and stored under water.

Preparation of Columns.—The column consisted of an 18-in. length of glass tube of 1-cm. i.d., fitted with a device at the bottom to regulate the flow rate and with an 80–100 ml. reservoir on the top. Glass wool plugs were used to support the resin bed and to cover the top of the bed. Twelve milliliters of wet cation exchange resin was slurried with water, added to the column, and allowed to settle by gravity. Twelve milliliters of wet anion exchange resin similarly was added on top of the cation resin. The column was washed with water immediately prior to use until suitably low assay blanks were obtained. Elutions were performed at a flow rate of 0.5–0.8 ml./min.

Separation Procedures.—*SRG Extraction.*—Dissolve 1.0 Gm. of sample in 25 ml. of chloroform and bubble nitrogen through the solution for 5 min.; extract twice with 10 ml. of distilled water. Combine the separated aqueous phases and dilute to 25 ml. with water. Wash a portion of this solution three times with equal volumes of chloroform and discard the chloroform phases.

TRG Extraction.—Weigh 1.0 Gm. of sample into a chilled 200-ml. pressure bottle. Rapidly add 10 ml. of chilled 1 *N* H_2SO_4 and 25 ml. of chilled chloroform and seal the bottle; shake on a mechanical shaker for 1–2 hr. at ambient temperature. Transfer the contents to a separator and separate the phases. Rinse the pressure bottle twice with 5 ml. of water and use this water to wash the chloroform phase. Combine the resulting aqueous phase with the initial 1 *N* H_2SO_4 phase and dilute to 25 ml. with water. Wash a portion of this solution three times with equal volume portions of chloroform.

SRG Ion Exchange.—Dissolve 1.0 Gm. of sample in 60 ml. of water. Bubble water-saturated nitrogen through the solution for 5 min., then add 10 ml. of 0.7 *N* H_2SO_4. Pass the solution through the ion exchange column. Discard the first 35 ml. and collect successive 5-ml. fractions of eluate.

TABLE VI.—INFLUENCE OF THE SAMPLE PREPARATION TECHNIQUE

Sample	Total Residual Glycol Found, %: Aqueous Acid Leach[a]	Complete Soln.[a]
Hydrocortisone *tert*-butyl acetate	0.14	0.41, 0.41
Prednisolone *tert*-butyl acetate	0.30	0.33

[a] Both heterogeneous systems (aqueous acid–solid sample, aqueous acid–chloroform) were shaken continuously during the hydrolysis step.

Establish that the eluate has reached a constant glycol concentration by assaying several successive fractions.

TRG Ion Exchange.—Transfer 1.0 Gm. of sample to a chilled 200-ml. pressure bottle; add 10 ml. of chilled 0.7 *N* H_2SO_4, seal immediately, and shake for 2 hr. at ambient temperature. Add 60 ml. of water, transfer the solution to the ion exchange column, and proceed as described above.

Colorimetric Procedure.—*Calibration Solutions.*—Accurately prepare a stock solution of glycol in water containing 20 mcg./ml. Pipet 0 (reference solution), 1, 2, 3, 4, and 5 ml., respectively, into 10-ml. volumetric flasks. Adjust the volume in each flask to about 5 ml. with water.

Sample Solution.—Transfer an aliquot of sample solution, containing about 50 mcg. of glycol in a volume between 1–5 ml., to a 10-ml. volumetric flask. Adjust the volume to about 5 ml. with water.

Oxidation.—Add 1 ml. of periodic acid reagent to each flask and mix. After 10 min., add 1 ml. of arsenite reagent and mix. Cool the solution in an ice bath and slowly add 2 ml. of cooled concentrated H_2SO_4. Bring the solution to room temperature and adjust to exactly 10 ml. with water. Extract a portion of the resulting solution several times with double volumes of carbon tetrachloride to remove the liberated iodine.

Color Development.—Pipet 2 ml. of each extracted solution into a 10-ml. glass-stoppered cylinder. Add exactly about 10 ml. of chromotropic acid reagent with a calibrated rapid flow pipet (*i.e.*, cut off the tip of a regular pipet). Heat in a water bath at 70° for 20 min. Cool to room temperature and determine the absorbance of each solution against the reference solution in 1-cm. cells at 580 mμ. Good temperature control during the measurement is required, since the temperature coefficient of absorbance of the chromotropic acid–formaldehyde adduct is about 0.9%/°C. (18). Construct a calibration curve and determine the glycol content in the samples by interpolation.

Results

Sample Preparation.—To determine the SRG content, the sample, after dissolution in a neutral solvent, is freed from any residual ETO by sparging its solution with nitrogen for 5 min. The complete dissolution of the sample eliminates the possibility of incomplete removal of ETO due to solvate bonds in the solid state, and the use of a neutral solvent minimizes hydrolysis of ETO before its removal. To determine the TRG content, the sample and a chilled acid solution are added to a chilled pressure bottle, which is immediately sealed. A pressure bottle avoids losses of ETO before and during hydrolysis. When necessary, chloroform is also added to the hydrolysis mixture to insure complete solution of the sample and thereby complete availability of the ETO and glycol. Failure to use the complete solution technique may lead to erroneously low results, as indicated by the data in Table VI.

Hydrolysis.—Although the hydration of ETO is subject to both acid and base catalysis (19), mild acid hydrolysis is usually more convenient and rapid (15, 19). Recoveries obtained after hydrolysis with 1 *N* H_2SO_4 for 1 hr., shown in Table VII, average about 97%. These results are in accord with the observation (20) that the use of sulfuric acid for the hydrolysis may lead to the formation of polymerized glycols and to results after periodic acid oxidation that are several per cent low. Higher recoveries could presumably be obtained (20) by use of perchloric acid instead of sulfuric acid.

Separation of Ethylene Glycol.—The products to be assayed consisted of two general types—nonpolar steroids and ionic antibiotics. Preliminary studies indicated that the presence of compounds from both groups in the solution to be assayed led to interference in the colorimetric reaction, either as a result of oxidation by periodate or by color formation in sulfuric acid. Accordingly, it was necessary to separate the glycol from the parent compound to perform the assay.

Extraction.—Glycol was removed from chloroform solutions of the steroid group (cortisone acetate, prednisone, prednisolone, *tert*-butyl acetate, hydrocortisone *tert*-butyl acetate) by extraction with water. The partition coefficient of glycol in this system appears to be <0.001, *i.e.*, it was found that one equal volume extraction was sufficient to remove glycol quantitatively from chloroform and that successive washing of an aqueous glycol solution with chloroform gave negligible losses. Similarly, studies of the extraction of the steroids in a chloroform–water system, followed either by U.V. absorption or by determining the residual mass after

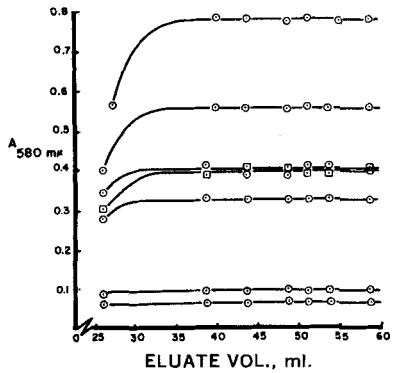

Fig. 2.—Typical elution curves in the ion exchange separation of ethylene glycol from a penicillin–streptomycin formulation. Curves are as follows (bottom to top): column and reagent blank; unsterilized sample (US); US + 0.076% glycol; duplicates (⊙ and ⊡) of a sterilized sample; US + 0.152% glycol; and US + 0.228% glycol.

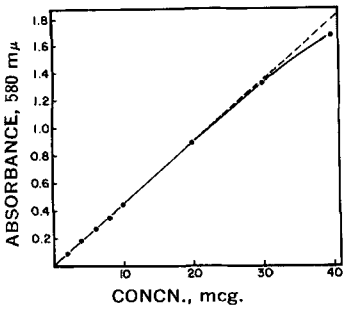

Fig. 3.—Colorimetric calibration curve. Concentration refers to the total micrograms in the final assay solution.

evaporation of the aqueous phase, indicated partition coefficients well above 100 in most cases. In general, three chloroform extractions were sufficient to remove the parent steroid as well as any trace impurities from the aqueous phase.

Ion Exchange.—Ionic components in the sample solution (either before or after the hydrolysis step) are removed by passing the solution through a two stage anion–cation resin bed under conditions giving quantitative recovery of the glycol. The anion resin is used in the first stage principally to neutralize the acid added in the hydrolysis step, thus facilitating the cation exchange in the second stage. Typical elution curves are shown in Fig. 2 for untreated and ETO–CO_2 (10:90) sterilized samples of an antibiotic formulation containing streptomycin sulfate, potassium penicillin, and procaine, among other components. After sufficient solution is added to displace completely the liquid initially present in the column, the eluate reaches a region of constant concentration. The glycol concentration in this region is the same as that in the input solution, but the solution is now free of ionic substances interfering in the colorimetric assay. In practice, the constant concentration region may be located readily by assaying several successive fractions of eluate.

Color Development.—The chromotropic acid reaction with formaldehyde in strong sulfuric acid (7, 17) was used as the basis of the colorimetric assay for glycol. Glycol is oxidized rapidly and quantitatively to formaldehyde with periodic acid (21), and the excess periodic acid is reduced with arsenious acid (10). The iodine liberated on acidifying the resultant solution is removed by extraction with carbon tetrachloride, leaving a formaldehyde solution suitable for assay.

A typical calibration curve is shown in Fig. 3. The absorbance is linearly related to concentration up to an absorbance of about 1. At higher absorbance values, where the ratio of chromotropic acid to formaldehyde decreases, the wavelength of maximum absorption (580 mμ) and the absorptivity at 580 mμ slowly decrease, resulting in a negative deviation from Beer's law. This is believed to be due to the formation of condensation species of higher polymerization, since dilution of the final solution once color development is complete is without effect on the new wavelength of maximum absorption or its absorptivity. The use of larger concentrations of chromotropic acid in the assay is not desirable, as the reagent blank increases with reagent concentration and with time. For these reasons, it is best to prepare fresh reagent immedi-

Table VII.—Recovery of Ethylene Oxide and Ethylene Glycol by Use of the Total Residual Glycol Method

Sample	Type Separation	Component Added[a]	Added, %	Recovery, %[b]
Prednisone	Extraction	Ethylene oxide	0.0010	101
Prednisone	Extraction	Ethylene oxide	0.0040	95
Prednisone	Extraction	Ethylene oxide	3.15	95
Prednisone	Extraction	Ethylene oxide	3.16	96
Prednisone	Extraction	Ethylene glycol	0.0080	98
Prednisone	Extraction	Ethylene glycol	0.0200	97
Prednisone	Extraction	Ethylene glycol	0.0200	96
Antibiotic formulation	Ion exchange	Ethylene glycol	0.076	99
Antibiotic formulation	Ion exchange	Ethylene glycol	0.152	99
Antibiotic formulation	Ion exchange	Ethylene glycol	0.228	98

[a] ETO was added by the Dumas Bulb technique; glycol was added as an aqueous solution. [b] Data corrected for sample and reagent blanks (prednisone, <0.0002%; antibiotic, 0.001%).

Table VIII.—Glycol Content of Carboxide Sterilized Pharmaceuticals

Sample No.	Substance	% Total Residual Glycol[a] Untreated[b]	% Total Residual Glycol[a] Sterilized[d]	Head Space Assay[e]
1	Prednisone	<0.0002	0.0027	+
2	Hydrocortisone *tert*-butyl acetate	0.010, 0.011	0.41, 0.41	+
3	Prednisolone *tert*-butyl acetate	0.012	0.33	+
4	Cortisone acetate	0.011, 0.013	0.92, 0.92	...
5	Cortisone acetate	<0.010[c]	0.90, 0.94	...
6	Cortisone acetate	...	0.80, 0.81	...
7	Cortisone acetate	...	0.61	...
8	Antibiotic formulation	0.0010	0.0109, 0.0110	...

[a] The extraction separation was used for all samples, except No. *8*, for which the ion exchange separation was used. [b] For all samples, except No. *5–7*, an aliquot of the sample subsequently sterilized was used. [c] Five individual samples from the same production run as the sterilized samples gave results within the range 0.003–0.008% glycol. [d] Results corrected for untreated sample content. [e] A + mark signifies that ETO was detected by GLC in the air space above sterilized samples stored in septum-sealed vials.

TABLE IX.—DISTRIBUTION OF TOTAL RESIDUAL GLYCOL IN PREDNISONE

Treatment	TRG, %	Free Ethylene Oxide, %
None	<0.0002[a]	<0.00002[a]
Sterilization	0.0027	0.0001
Sterilization	0.0016, 0.0019	0.00005

[a] Detection limit for these assays.

ately before use, to add the reagent to the calibration and test solutions, as well as make the absorbance measurements, at approximately the same elapsed time, and to dilute the solutions to be assayed, before color development, to fall within the linear calibration range. With these precautions, the precision of each intraday calibration series was consistently within 2%, although the slope of the calibration curve varied within a 10% range on a day-to-day basis.

Precision and Accuracy.—Results of recovery experiments, shown in Table VII, indicate that the over-all accuracy of the two modifications of the TRG method is at least 95%. Precision, indicated by the average deviation of spot checks of individual samples in Tables VII and VIII, is in the order of ±2%. The lower limit of sensitivity of the method described is about 0.0002%. Greater sensitivity may be obtained readily, however, by scaling up the concentration factors at appropriate points in the procedure.

Results.—The results in Table VIII indicate that ETO–CO_2 sterilization leaves a total glycol residue in all of the samples tested. In some cases, almost a 1% residue was found. The level of total glycol to be found in any given sample at any given time and the distribution of total glycol between ETO and glycol is undoubtedly a function of the post-sterilization age of the sample and the conditions of storage. Distribution of total glycol was investigated in one case, with results listed in Table IX. Here, only about 3% of the TRG was present specifically as ETO. The presence of residual ETO in other compounds may be inferred from the GLC head space assays listed in Table VIII.

DISCUSSION

It is clear from the diversity of results obtained in this investigation that the content of reversibly bound residual ETO and glycol must be studied on an individual basis for each type of sample. Irreversibly bound residues, *i.e.*, residues based on chemical reaction between ETO or glycol and the sterilized substance, are beyond the scope of this work since they would not generally respond to the assays used here. Although such reactions are known to occur with amino acids and some pharmaceuticals (1, 16, 22, 23), a paper chromatographic study of sterilized cortisone acetate did not indicate alteration of the parent compound or the formation of new components.

Before effective means of reducing residues to permissible tolerances can be developed, the mechanism of retention must be understood. Although the general importance of sorption phenomena has been recognized (4), little is known about specific mechanisms. The data obtained in this study permit several hypotheses to be advanced.

Sorption–Liquid Phase.—The presence of any residual solvent in the sample, *i.e.*, occluded mother liquors, incompletely dried or hygroscopic samples, etc., may lead to absorption of ETO. The extent of absorption would depend, among other factors, upon the solubility of ETO in the liquid phase and the concentration and duration of exposure. Water is a solvent of particular importance in this respect, not only in view of its high sorptive capacity [up to 0.4 Gm./(Gm. H_2O) at 30° (3)] but also in view of its participation as a reactant in the hydrolysis of ETO. Samples of high water content would thus be expected to show high initial retention of ETO and correspondingly high levels of glycol after storage.

The results for cortisone acetate wet cake (~60% H_2O) best exemplify the behavior of this sample category. Cortisone acetate does not contain specific residual ETO, yet it has the highest residual glycol content of any of the samples tested. Although the rate of hydrolysis under the given treatment and storage conditions is not known, other similarly treated steroids, containing considerably less water, retain at least some of their residues as specific ETO when stored for the same length of time. With respect to the rate of hydrolysis, the carbon dioxide component of ETO–CO_2 gas sterilization formulations is potentially detrimental. The sorption of CO_2 by the liquid water phase may lead to an increase in acidity which would accelerate hydrolysis.

Sorption–Solid Phase.—It has been reported (24) that ETO is only slowly released from sterilized plastics. A similar effect has been noted here for several sterilized crystalline steroids (*cf.* Table V, *Footnote b*). Although the nature of these materials is quite different, the common feature of their behavior appears to be that the concentration gradient responsible for diffusion is determined by the strength and nature of the ETO–solid interaction. The interaction is of the solubility type for the plastics (25) and is presumably adsorptive for the steroids.

ETO would not be expected to diffuse appreciably into solids present as well-defined crystals. In general, ETO would not fit within the crystal lattice, and the energy involved in the diffusion process, barring specific solubility interactions, would not be sufficient to break the lattice bonds. The absence of such penetration may well be the cause of the occasional failure of ETO to sterilize crystalline samples (2).

Adsorption of ETO on the surfaces of the sample is undoubtedly a major mechanism for its retention and must for the present be considered as a phenomenon specific to the type of surface available to the vapor. Solvation, however, is one type of adsorption interaction warranting individual mention. Table V shows that, of the types of compounds analyzed, appreciable retention of specific ETO is found only among the steroid group, and even here it is limited to particular members of the group. Thus, the *tert*-butyl esters of prednisolone and hydrocortisone show appreciable retention, whereas hydrocortisone acetate and prednisone show only trace retention. These differences may be rationalized by consideration of solvation phenomena.

Many steroids are well known to be capable of solvation, and various specific states of solvation are often associated with individual crystallographic forms. In the present work, X-ray diffraction studies indicated that the two steroids showing trace retention of ETO were submitted to sterilization in unsolvated crystal forms, whereas the two steroids showing appreciable ETO retention were sterilized in solvated (principally hydrated) forms. In both the latter cases, however, the samples were incompletely solvated and possessed additional capacity equivalent to at least 0.5 of a monosolvate. The residual ETO content of these samples, amounting to less than 0.1 of a monosolvate, thus could be accommodated readily on accessible solvation sites. Mixed monosolvate formation has been observed previously by the author for compounds crystallized from mixed solvents and is not an isolated phenomenon.

Mechanism of Glycol Retention.—Glycol is a secondary residue, resulting from the hydrolysis of ETO. The presence of ETO in the head space over samples stored in closed containers indicates that it is sufficiently mobile to react with water in the vapor phase (26) as well as within or on the samples.

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Interactions of Xanthine Derivatives with Bovine Serum Albumin II

Spectrophotometric Studies

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The spectral characteristics in aqueous solution of a number of xanthine derivatives were found to be influenced by the presence of bovine and human serum albumin. The observed effects were assumed to be due to the binding of the xanthines by the protein. Changes in spectra were thus used as the basis for an experimental method for determining the extent and nature of the small molecule-protein interaction. The effects of temperature, pH, and protein modification on binding behavior were studied. Comparative studies on a number of structurally different xanthines were conducted. The results indicate that a rather specific orientation of the protein molecule was required for optimal interaction. Expansion or other configurational changes induced by pH or temperature effects resulted in decreased binding. The binding site appears to contain a grouping which can hydrogen-bond with the xanthine. The participation of other types of interaction forces are suggested also.

A PREVIOUS REPORT (1) communicated the results of a study in which the binding of various xanthines by bovine serum albumin (BSA) was investigated by an equilibrium dialysis technique. By utilizing this experimental method, it was possible to demonstrate that a number of xanthines were bound reversibly by BSA, that the binding was pH dependent, and that structural modification of the small molecule had a pronounced influence on the degree and nature of the binding. It was the purpose of this investigation to restudy the previously investigated systems by a spectrophotometric

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