AN ANALYTICAL METHOD FOR THE DETERMINATION OF A DIALKYL DIMETHYL QUATERNARY AMMONIUM CHLORIDE IN BEEF LIVER TISSUE

by

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INTRODUCTION

Bloat Background - Bloat is a condition in beef and dairy cattle which is characterized by an increase in rumen fluid viscosity (1). This condition causes fermentation gases to become trapped in a tenacious foam. This foam, as it increases in volume, impairs eructation and distends the animal's abdomen. The distention can become severe enough to prevent breathing and the animal suffocates.

There are two kinds of bloat - pasture and grain bloat. Pasture bloat is primarily a result of substances found in plants with a small contribution from microorganisms. Grain bloat is caused mainly by the action of the microorganisms in the rumen (2). The occurrence and severity of grain bloat has been correlated with increased slime encapsulated rumen bacteria counts (4,5). Experimental evidence (2) has shown that large numbers of long chains of streptococci are found in the rumen contents of cattle exhibiting grain bloat. It is thought (1) that the capsular material around the bacteria increases rumen viscosity, thereby, improving its ability to trap fermentation gases. The capsular material has been found to consist primarily of rhamnose, galactose, and galacturonic acid (6). Pasture bloat can be controlled by the addition

of poloxalene (2) (a polyoxyethylene - polyoxypropylene block polymer) to the animal's diet. Grain bloat, however, has no commercially available drug in the U.S.A. to prevent it (2).

The effect of grain bloat in cattle is significant. In Kansas a survey of feedlots feeding approximately 450,000 head and having between 200 and 95,000 head per lot was taken (3). Results showed that 0.1% died of bloat, 0.2% had severe cases of bloat and 0.6% had some degree of bloat. In the opinion of 47% of the 60 operators, bloat was an important economic problem. Seventy-seven % felt that fatality due to bloat was the most significant economic loss from bloat. If one can consider the death loss due to bloat in Kansas representative of similar losses in the rest of the United States, then the average annual loss due to bloat would exceed eight million dollars.

When cattle on grain feed bloat to less than a fatal degree they will either reduce their feed intake or go off feed entirely. This reduces the rate of weight gain of beef cattle which results in an increased period of time before the cattle are brought to market weight. With dairy cattle, reduced feed intake can result in decreased milk production (7). The economic impact of bloat induced reduction of feed intake is difficult to measure because there is no commercially available grain bloat preventative.

After screening 235 drugs, Meyer and Bartley (8) have

found that a water soluble dimethyl dialkyl quaternary ammonium compound was effective in the prevention of grain bloat in beef and dairy cattle. Nagaraja, Bartley, and Fina (9) have found that the action of the quaternary ammonium compound is to inhibit the production of frothy slime. This bacterial slime is responsible for the entrapment of fermentation gases. The quaternary ammonium compound used to prevent grain bloat is dialkyl dimethyl ammonium chloride, hereafter, referred to by the manufacturer's trade name Arosurf TA-100. See page 13.
For this compound to become a commercial veterinary drug for animals, which are ultimately consumed by humans, the Food and Drug Administration has made two stipulations: (10) 1. that there must be a method for determination of the compound in the tissue of the animal at levels of 2-5ppm in order to obtain a preliminary approval for field testing the drug and 2. a method to about 0.1ppm to accompany the new drug application. This paper describes and discusses the development of an analytical method for the determination of Arosurf TA-100 in the presence of beef liver.

Analytical Approach - The liver was selected as the tissue to analyze for the drug content because, if the compound is metabolized, the liver will be a very likely place to find it. The fact that the liver acts as the major clean up or detoxification organ of the body (85% of foreign chemicals are detoxified here) makes it a likely place to find the drug. This is especially important

when the drug is withdrawn from the feed, prior to slaughter, because the liver is most likely the tissue which maintains the highest concentration of the drug during the animal's withdrawal period.

The question of whether the quaternary amine will remain unaltered during metabolism cannot be completely answered until feeding studies are done. Work by Bartley et al. (11) which investigated an extensive number of extraction/sample treatment (203/8) systems proved to be successful in performing analyses on drug liver mixtures of 5 ppm or more on samples of liver spiked with drug. The analytical methods for measuring how much of the quaternary salt was extracted were colorimetric and chemical fluorescence techniques. The colorimetric technique used bromphenol blue as color developing agent. To a liver spike extract, containing the quaternary cation, was added bromphenol blue. The amine dye complex was then extracted into ethylene dichloride and the absorbance was measured at 606 nanometers. The fluorescence procedure involved forming a Eosin yellow/quaternary cation complex in a liver spike extract and measuring the fluorescence with excitation and emission wavelengths of 325 and 560 nanometers respectively.

Apparently, small amounts of the quaternary amine are tightly held to the liver or, perhaps, to albumin from the dried blood left in the liver (12). In an attempt to overcome this, two approaches were tried. One was to chemically react the quaternary amine to break off a

chemically measurable part and the second was to attempt to pull the quaternary amine away from whatever was holding it by forming a dye amine complex and then extracting that neutral species into an organic solvent.

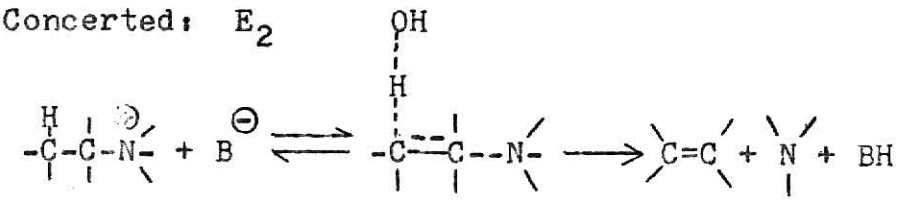
Theoretical Basis for Present Work - Quaternary amines can be degraded by elimination of the nitrogen atom and a beta hydrogen atom to form an olefin and a tertiary amine (13). This reaction was reported by Hoffmann in 1851, and bears his name (14, 15).

$$(RCH_2CH_2)(R')_3\overset{+}{N} \xrightarrow{\overset{-}{OH}} RCH{=}CH_2 + R'_3N$$

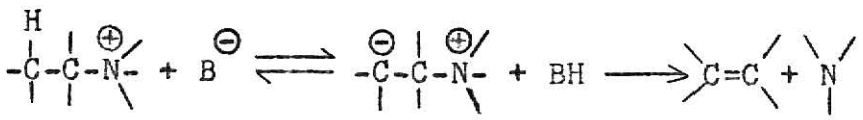
The olefin can then be looked for in very small amounts by Gas Liquid Chromatography with a flame ionization detector.

Mechanism - The mechanism of elimination can be E_2 or E_1 in character, but it is usually some degree of both.

Concerted: E_2



Stepwise: E_1



The transition state acquires more carbanion character as the beta hydrogen atom becomes more acidic. The same trend occurs as stronger bases are used (11). In order

for a quaternary amine to undergo degradation in this manner, four requirements must be met:

1. a strong base must be present,
2. the molecule must have a hydrogen atom on the carbon beta to the nitrogen atom,
3. the nitrogen atom must be positively charged,
4. sufficient energy must be supplied for the particular molecule to decompose.

For alkyl quaternary ammonium compounds, there is evidence (16) that the elimination mechanism is essentially E_2. Shiner and Smith (16) have shown that during reaction in deuterated solvents alpha hydrogens were exchanged with deuterium while beta hydrogens were not. This shows that any carbanion character which may exist in the transition state is very short lived and that the olefin forms faster than deuterium substitution can take place. The beta hydrogen has been shown to be involved in the rate determining step because when it is deuterium instead of hydrogen, the rate is decreased approximately four fold.

The type of olefin which is formed is controlled by three factors. They are as follows:

1. the amount of stabilization the olefin gets from conjugation or hyperconjugation,
2. the acidity of the beta hydrogen which is being eliminated,
3. steric interactions in the transition state by surrounding groups.

Competing Reactions - The formation of alcohols instead of olefins is the most important competing reaction in the degradation of quaternary amines. It results from attack by hydroxide ion on the alpha carbon instead of the beta hydrogen. The products are an

alcohol and tertiary amine instead of an olefin and tertiary amine.

$$1.\quad (R)_2\overset{+}{N}(CH_3)_2 + OH^- \longrightarrow (R)_2NCH_3 + CH_3OH$$

$$2.\quad (R)_2\overset{+}{N}(CH_3)_2 + OH^- \longrightarrow RN(CH_3)_2 + ROH$$

One cannot avoid some alcohol production because the rate of the reaction which produces the alcohol is enhanced by the same factors which enhance the rate of olefin production. Alcohol formation may predominate over the olefin formation if anions which are less basic than hydroxide or alkoxide are used such as acetate, carbonate, or phenoxide (17). If no benzyl or allyl groups are present in the quaternary amines, which is the case with the quaternary amine under study, then reaction 2. is preferred over reaction 1.

Methods to Consider - The Hoffmann elimination reaction can be brought about in a variety of ways. The method most commonly used (13) is to convert the quaternary salt to the hydroxide with silver oxide. The silver halide is removed by filtration and excess water is driven off by mild heating. The temperature of the resulting syrup is then raised until elimination occurs.

$$(R)_4\overset{+}{N}Cl^- \xrightarrow{Ag_2O} (R)_4\overset{+}{N}OH^- + AgCl \xrightarrow[heat]{} \text{elimination}$$

This approach is not applicable to the task at hand because the quaternary amine being degraded is adsorbed

on liver tissue and, therefore, concentration to a syrup is not possible.

The second most commonly used (13) method is to simply heat the quaternary amine salt with concentrated excess base to effect elimination.

$$(R)_4\overset{+}{N}Cl^- \xrightarrow[\text{heat}]{\overset{-}{OH}} \text{elimination}$$

This method is the basis for this experimental work. The pyrolyses can be done in small sealed vessels or under aspirator vacuum to reduce atmospheric CO_2 introduction. Or the reaction could be carried out in a CO_2 free atmosphere such as nitrogen.

Although bases stronger than hydroxide may be used, experimental evidence has shown that no improvement in yield of of olefin is realized (18).

Drug-Dye Englomerate Formation - Salts of low charge/size ratio exhibit very low aqueous solubility (19). Such a salt would be an alkyl quaternary ammonium cation and large organic dye anion combination.

It was felt that, if a sample of liver and drug was treated with an organic dye in its dissociated form, perhaps, an englomerate salt would be formed.

$$\overset{+}{R_4N} + Dye^- \longrightarrow R_4N\text{-}Dye$$

This species would then be extractable into an organic solvent. The absorbance of the extract, when measured,

should be a measure of the amount of drug originally in the liver sample. When these results are compared with the amount of dye-drug englomerate extracted from a sample containing no liver, the effectiveness of the dye anion in pulling the drug away from the liver can be deduced.

EXPERIMENTAL

Apparatus - Three types of reaction vessels were used. They were as follows:

1. stainless steel bombs with teflon liners,
2. five milliliter hard glass lypholization ampules,
3. 30mm x 250mm cold traps with 24/40 ground glass joints.

See Figure I on page 11.

The chromatographic work was begun on an Aerograph model 500 Hi-Fi and work was eventually shifted to a Bendix 2200 when it became available for use. The chromatographic column was a 1/8" outside diameter thin wall stainless steel column 5 feet long packed with 3% Dexsil 300GC on 90/100 anakrom SD. A flame ionization detector was used with both instruments. Column conditions were as follows:

1. Helium flow rate 16 milliliters per minute (soap bubble method),
2. Oven temperature was programmed from 140°C to 220°C at 16° per minute with a 2.5 minute hold at the start.

A Kuderna-Danish concentrator was used to concentrate the reaction products in hexane. The bombs were obtained from Uni-Seal Decomposition Vessels Ltd. The Dexsil 300GC packing material was obtained from Analabs, Inc.

Reagents - One gram of 99% pure octadecene was obtained for the preparation of standard solutions in

FIGURE I

APPARATUS FOR ELIMINATION PYROLYSES

A. REFLUX METHOD

B. AMPULE METHOD

C. BOMB METHOD

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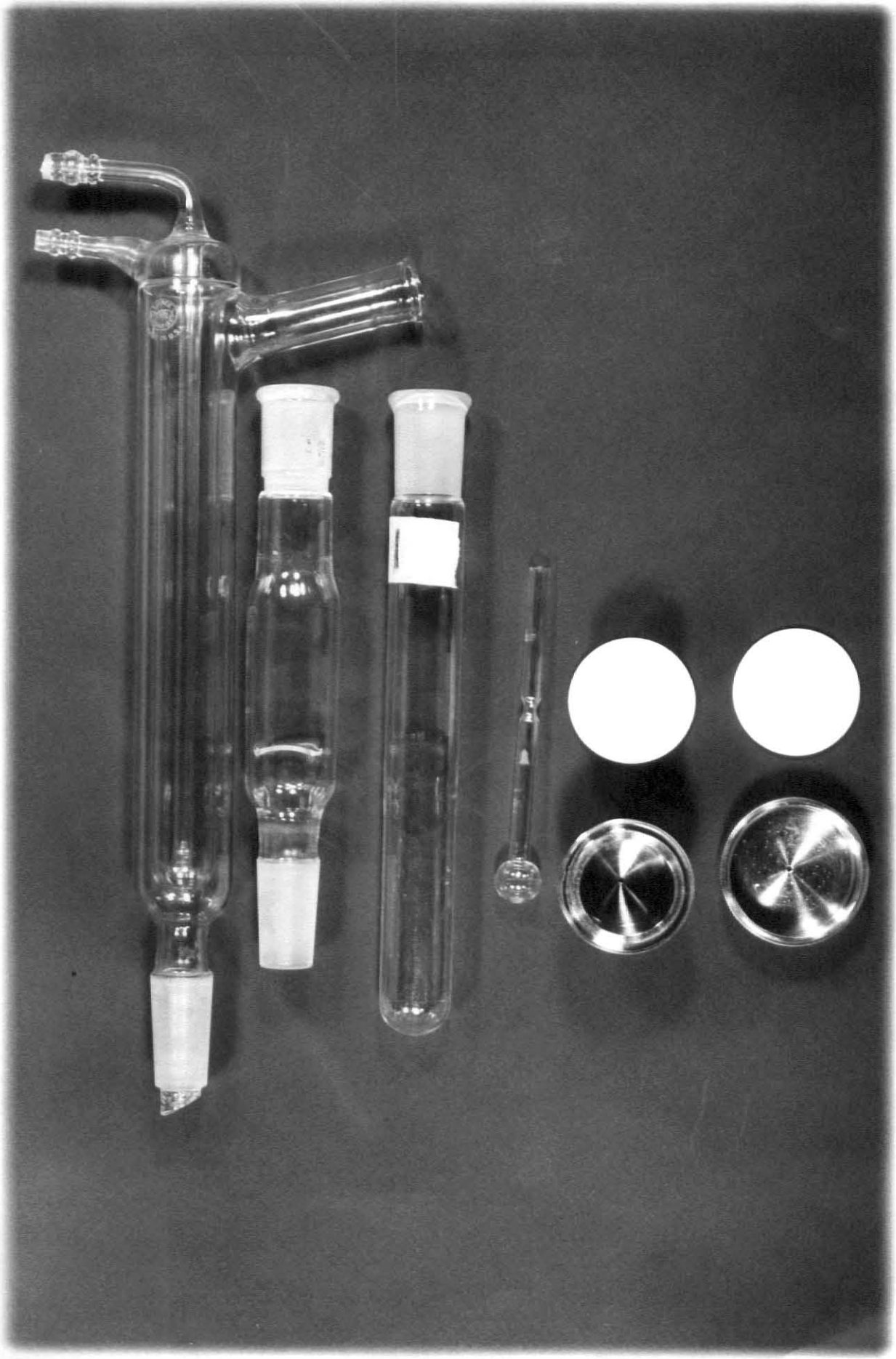

hexane. These solutions were used as a reference for quantitation of octadecene yields. A mixture of a homologous series of hydrocarbons consisting of tetradecane and tetradecene through eicosane and eicosene omitting the seventeen carbon pair was used to test how well the column would separate compounds similar to octadecene. These two reagents were obtained from Applied Science Laboratories, Inc.

Chromatographically pure hexane was used to dissolve the Hoffmann reaction products and to extract the dye-drug englomerate. It was obtained from Mallinckrodt, Inc. and was their "Nanograde" grade of hexane. The liver used was obtained in a grocery store, freeze-dried, and homogenized in a blender. Whatman IPS phase separation paper was used to filter the hexane solution of the Hoffmann reaction products. Ten percent hexamethyldisilazane in hexane was used to coat all glassware, except the reaction vessels, which came into contact with the quaternary ammonium salt. The hexamethyldisilazane was obtained from Applied Science Laboratories, Inc. Orange II, fast red S, and bromphenol blue were used in the dye extraction procedure. These dyes were stock items. See Figure II on page 14 for dye structures.

Manufacturer's Specifications (20) - The drug is produced by Ashland Chemical Company, Box 2219, Columbus, Ohio 43216. Its registered trade name is Arosurf TA-100. It is a cationic surface active material which can be used with nonionic surfactants and other quaternary

FIGURE II

STRUCTURES OF DYES DISCUSSED

A. ORANGE II

B. FAST RED S

C. BROMPHENOL BLUE

D. EOSINE YELLOW

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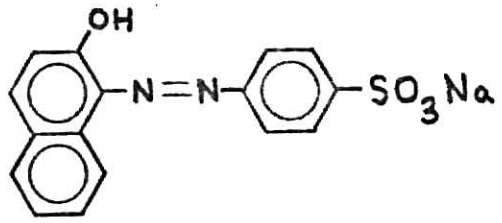
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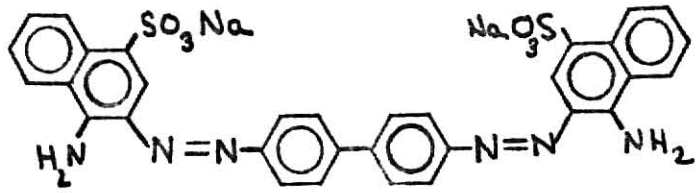
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N=N
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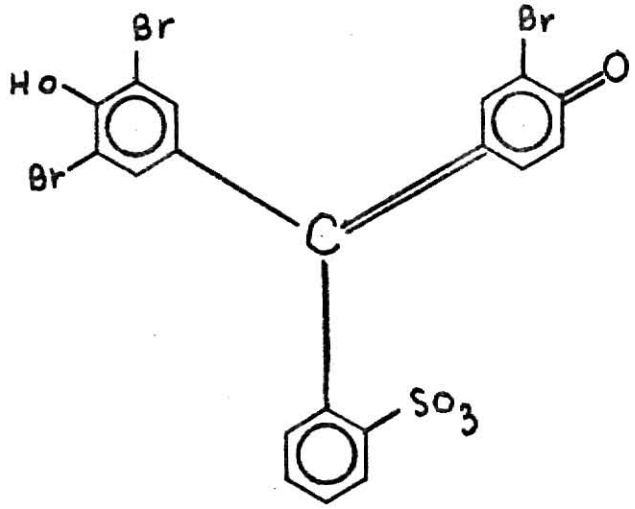
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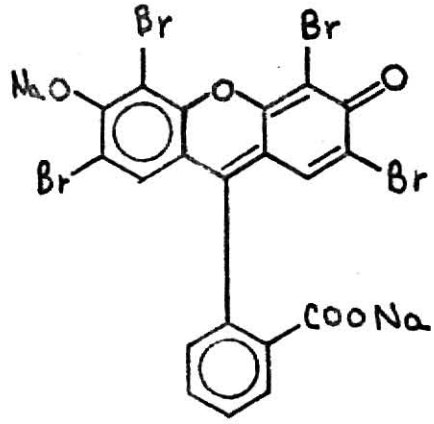
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Br
NaO
O
Br
Br
COONa

product. The average molecular weight of the product is 583.

Procedure - Pyrolyses were run on three types of samples:

1. liver plus quaternary salt - liver spike
2. liver without quaternary salt - liver blank
3. quaternary salt without liver - quaternary blank

Reagents used to decompose the quaternary ammonium salt were potassium hydroxide and silver oxide.

Bomb Method - Into the teflon liner were placed base, a quantitative amount of quaternary ammonium solution, and liver in liver spikes. Silver oxide was generated in situ from silver nitrate. When silver oxide was desired in the reaction, base was always added after silver nitrate was mixed throughout the reaction mixture. In this way a fine particulate dispersion of the oxide is obtained as opposed to a few large lumps when the silver nitrate is added after the alkali. The pyrolysis was then carried out in an oven. Maximum temperature allowed was 180° C per manufacturer's specifications. Products work up will be discussed under the ampule method.

Ampule Method - Glass ampules were the next choice of reaction vessel because they could be sealed and heated to approximately the same temperatures as the bombs. The ampule maximum temperature was 150° C. Above that temperature, they tended to burst. Pyrolyses were run on samples using 8N potassium hydroxide, saturated potassium hydroxide, or a mixture of potassium hydroxide and silver

ammonium compounds such as germicides. Its manufacturer-suggested uses are as a laundry additive for the purposes of fabric softening, faster water extraction during spinning, faster drying, minimization of lint formation, easier ironing, and a reduction in static electricity. According to the manufacturer, the supplied material is 95% active ingredient.

Active ingredient is defined as any alkyl quaternary amine. The product is a mixture of alkyl quaternary ammonium chloride salts. The alkyl constituents are primarily straight chain moieties of 14 to 22 carbons in length. The majority of these alkyl groups are 18 and 16 carbon units which are present in a 65:35 ratio. The following types of quaternary amines are found in the product:

1. tetramethyl $(CH_3)_4\overset{+}{N}Cl^-$
2. monoalkyl $(R)(CH_3)_3\overset{+}{N}Cl^-$
3. dialkyl $(R)_2(CH_3)_2\overset{+}{N}Cl^-$
4. trialkyl $(R)_3(CH_3)\overset{+}{N}Cl^-$
5. tetraalkyl $R_4\overset{+}{N}Cl^-$

The product is assumed to be 70% dialkyl amine and the ratio of trialkyl to dialkyl is known to be 84:26. Workers at Ashland Chemical Company (21) have found that the tetraalkyl and tetramethyl moieties do not undergo elimination and comprise less than or equal to 10% of the

oxide both in large excess. The quantity of each reactant was varied over the ranges as indicated below:

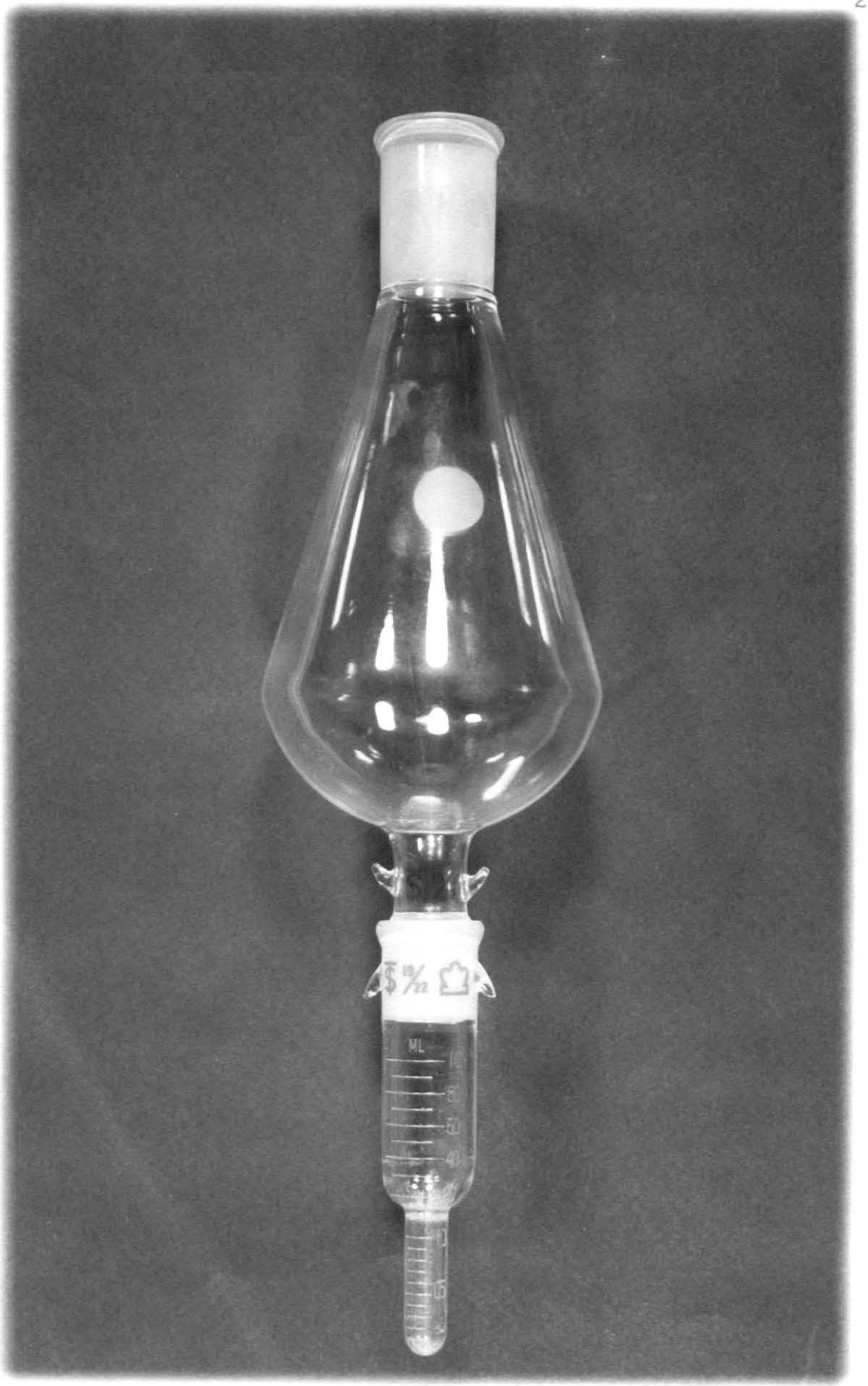
1. KOH 0.2 to 10g
2. Ag_2O 0 to 1 grams
3. Liver 0 to 2 grams
4. Drug 10 to 1000 micrograms

The ampules were prepared for reaction in the following way. The quaternary ammonium salt in solution was pipeted into the ampule followed by base. Liver was added first in liver spikes. The ampule was then sealed and heated for a measured period of time. After cool down, the ampule was broken and the reaction products were washed out with hexane taking care to wash both pieces thoroughly. The olefin was found to condense in the upper portion of the ampule, so that part must not be neglected. The hexane solution of the reaction products was filtered through phase separation paper and the filtrate collected in a Kuderna-Danish concentrator. Atop the Kuderna-Danish concentrator is a simple foam trap to prevent the solution of products from spurting out. See Figure III on page 19. It also provides a small amount of reflux which is all that was felt necessary to prevent loss of the higher boiling octadecene. After the solution was concentrated to 0.5 milliliters, a sample was analysed for octadecene content by gas chromatography.

Reflux Method - Pyrex tubes, which were actually the bottom half of a cold trap, were used to run the reaction under hexane. A photograph of this apparatus can be found on page 11. Atop the trap tube was affixed a foam

FIGURE III

PHOTO OF A KUDERNA-DANISH CONCENTRATOR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trap and a cold water condenser. The reactants in the tube were the same as in the ampules with the following exceptions. Fifty milliliters of hexane covered the aqueous contents and prevented atmospheric carbon dioxide from getting into the reaction. After a considerable number of pyrolyses were run with a hexane reflux, a number were run without hexane present. This raised the reaction temperature from 70° C to 98° C. Due to the improved results and no apparent problems from carbon dioxide, (see Table I), the reactions were run without a hexane reflux for the remainder of the investigation. A small amount of methanol, usually a milliliter or less, was used to wet the liver. Without this, the aqueous solution of the quaternary salt would bead up on the surface of the liver. It was felt that this would not realistically simulate a liver quaternary homogenate. Boiling water and oil bath were used to heat this reaction system.

Reflux Product Work Up - Upon completion of the pyrolysis hexane was introduced into the reaction mixture and allowed to reflux for five minutes. After cooling, the hexane was decanted off and passed through Whatman IPS phase separation paper into a Kuderna-Danish concentrator. Three additional 30 milliliter portions of hexane were used to wash the reaction products and insure that the octadecene generated is quantitatively transferred to the Kuderna-Danish concentrator. The combined hexane extract is then concentrated to 0.5 milliliters. This requires

TABLE I

YIELD RESULTS OF HEXANE FREE REFLUX METHOD

COMPARED TO A TYPICAL YIELD FROM

A REFLUX CONTAINING HEXANE

DRUG LEVEL (ppm)	t (hr.)	T (deg. C)	HEXANE PRESENT	OLEFIN YIELD (%) Blank/Spike
100	8	70	yes	124/125
10	7.5	97	no	154/149
1	10	97	no	153/134

about 15 to 20 minutes with boiling water as the heat source. Gas chromatographic analysis for octadecene was then performed on this concentrate.

Dye Extraction Method - Experiments with the three dyes were carried out as described below:

BLANK	TEST
3ml H_2O	3ml 1000ppm TA-100
4 drops 8N KOH	4 drops 8N KOH
2 drops dye (sat.)	2 drops dye (sat.)
3ml hexane	3ml hexane

LIVER TEST
200mg liver
3ml 1000ppm TA-100
4 drops 8N KOH
2 drops dye (sat.)
3ml hexane

Reagents were added in the order in which they are listed and hexane was not added until complete mixing of the aqueous components was achieved. Upon addition of hexane with mixing, dye color transfer to the organic phase was observed.

Calculations - The amount of liver present in the reaction was worked up to 200 milligrams. The reason for this amount was that in future feeding studies, one gram biopsies could be performed without sacrificing the animal. Liver

is on the average 20% dry matter. Therefore, 200 milligrams of freeze-dried liver was used to simulate a one gram biopsy. In this way, when 100 micrograms of quaternary salt placed in a reaction mixture containing 200 milligrams of liver, it is simulating hydrated liver that is 100ppm in the quaternary salt. If 70% of the product is a dialkyl moiety and the ratio of dialkyl to trialkyl moieties is 84:26 then 83% of the product is composed of dialkyl and trialkyl moieties. Furthermore, since 10% or less of the product consists of tetramethyl and tetraalkyl moieties then approximately 7% of the product must be a monoalkyl moiety. Therefore it is assumed that 90% of the product can potentially undergo elimination in the Hoffmann reaction. The percentage of 14, 15, 17, 19, and 20 carbon alkyl moieties in the product is very small but not known exactly. The alkyl groups will be assumed to be 98% 18 and 16 carbon moieties. Therefore the maximum yield possible of octadecene is 0.25 grams per gram of TA-100. When the product was ready for analysis it was contained in 0.5 milliliters of hexane. See Results and Discussion for detection limits of octadecene with flame ionization detector used. All gas chromatographic peak areas were calculated as the product of height of the peak and the width of the peak at half height. Yield of olefin was determined by comparing the area of a peak obtained from the injection of a quantitative standard of octadecene with the area of the octadecene peak obtained from

reaction products. Standard and experimental injections were always the same volume. Maximum injection size was 8 microliters. Greater volumes overloaded the column.

RESULTS AND DISCUSSION

The dye extraction procedure results are listed in Table II on page 28. Apparently, the dye anions which were tried do not have a strong enough attraction for the quaternary cation to pull it away from the liver.

The teflon-lined steel bomb method initially looked promising in that similar yields of octadecene were obtained from reactions with and without liver present. See Table III on page 30. Liver blanks did not yield any peak of the same retention time as octadecene.

The reaction products, however, contained a hexane soluble residue that ruined a column. The problem was attributed to teflon liners because the damaging residue was not present in the products of pyrolyses run in glass vessels. Therefore, the bombs were set aside. A different column packing material may have been unaffected by the residue, but glass reaction vessels were used rather than obtaining new column materials.

Switching to glass ampules eliminated the column destruction problem, and since they could be sealed, they kept out atmospheric carbon dioxide. Octadecene yields from Hoffmann reactions containing no liver were maximized with only 0.5 milliliters of 8N potassium hydroxide and 0.5 milliliters of 0.1N silver nitrate, 90 minutes

TABLE II

DYE METHOD RESULTS

	DYE	DRUG (mg)	LIVER (mg)	DRUG/DYE TRANSFER TO HEXANE PHASE
BLANK	O II	0	0	no color transfer
	FRS	0	0	no color transfer
	BPB	0	0	no color transfer
TEST	O II	1	0	nearly complete color color transfer
	FRS	1	0	complete color transfer
	BPB	1	0	poor color transfer
LIVER TEST	O II	1	200	no color transfer
	FRS	1	200	no color transfer
	BPB	1	200	no color transfer

ORANGE II - O II

FAST RED S - FRS

BROMPHENOL BLUE - BPB

TABLE III

BOMB METHOD

BLANK/SPIKE YIELD COMPARISON

	DRUG LEVEL	YIELD OF OLEFIN
LIVER BLANK	100ppm	54%
LIVER SPIKE	100ppm	46%

reaction time and 150° C reaction temperatures. This maximum yield was approximately 56%.

When liver was introduced into the pyrolyses, 0.5 milliliters of 8N potassium hydroxide was no longer sufficient. Results obtained indicate that in order to effect the elimination reaction all of the liver tissue must first be destroyed. The reaction yield of 56% was never reached with liver present under any conditions in ampules. The reason for this is assumed to be that the maximum amount of potassium hydroxide which could be placed in the ampule was insufficient to completely solubilize the 200 milligrams of freeze-dried liver. The data in Table IV indicate how olefin yield increased in liver spikes as potassium hydroxide content was increased. Table IV is on page 33. The ampule would hold a maximum of three grams of potassium hydroxide plus liver, drug solution, and silver oxide. However, with these large amounts of potassium hydroxide, reproducibility was very poor due in the most part to the lack of mixing. The lack of mixing was suspected to be due to an inadequate supply of water. Since hydroxide was at saturated levels in the reaction vessel long before 3 grams were present, the additional amount of base, apparently, is needed to decompose the liver tissue. To make it available, adequate mixing must be possible. A larger reaction vessel had to be obtained.

It must be pointed out that it was not possible to quantitate these results due to instrumental problems. The

TABLE IV

RELATIONSHIP OF OLEFIN YIELD TO THE QUANTITY OF ALKALI PRESENT IN LIVER SPIKE REACTIONS RUN AT 150° C FOR 90 MINUTES IN GLASS AMPULES

DRUG (mg)	KOH (g)	Ag_2O (mg)	OLEFIN (units of peak height x attenuation)
2	0.2	20	16 x 4
2	0.3	20	20 x 4
2	0.4	20	22 x 4
2	0.5	20	30 x 4
2	0.6	20	38 x 4
2	1.0	20	50 x 4
2	1.3	20	62 x 4

response of the Aerograph unit was extremely nonlinear. Attempts to have the instrument repaired proved nonproductive. For these reasons and the fact that if only 56% yields could be achieved with liver spikes, the assay limit ultimately would be 200ppm, a new reaction system was sought.

It was at this point that an attempt was made to run the pyrolysis in a test tube at boiling water bath temperatures. The first rough reaction was run on 5 milligrams of the quaternary salt with 5 milliliters of 8N potassium hydroxide. Elimination was accomplished and further investigation of this method showed it to be a promising method.

The temperature limitation was no problem. The time allowed for reaction was increased accordingly. It compensated for the lower temperature nicely. Some attractive advantages are inherent in this method. They are as follows:

1. potentially unlimited sample size
2. ease in handling of products
3. essentially unlimited capacity for base

Switching to the reflux method under hexane gave an immediate improvement in olefin yield compared with the ampule method. This can be seen by comparing the results of the two reactions in Table V on page 36. The possibility that liver degradation could be yielding a compound that has a retention time the same as octadecene was investigated. Two hundred milligram samples of liver were degraded with base and treated in a manner identical to a

TABLE V

COMPARISON OF OLEFIN PRODUCTION

IN AMPULES VERSUS THE REFLUX METHOD

	DRUG	t min	KOH (g)	Ag_2O (mg)	T (C)	OLEFIN (units peak height)
AMPULE	1mg	90	3	20	150	23
REFLUX	1mg	90	3	20	70	52

liver spike. On no occasion did any peak resulting from a component contained in the liver degradation components coincide with the retention time of octadecene.

Pyrolyses with silver oxide present were, after some investigation, found to be unpredictable. Jennings and Mitchner (22) found that while the use of silver oxide as a base gave some good yields of olefin, potassium hydroxide by itself gave much more consistent results. When silver oxide was deleted from the reaction mixture, results became much more consistent. Once again, no interfering peaks from liver were found. The data in Table VI on page 39 are an example of the reproducibility of the reaction without silver oxide.

When an oil bath was used as the heat source for the reflux method no advantage was observed. Boiling water baths were more convenient. They were, therefore, used as the heat source for the remainder of experimental work.

When gas chromatographic analysis of the reaction products was shifted to the Bendix 2200 instrument, absolute detection limits were lowered from 20 nanograms of octadecene to 5 nanograms of octadecene. The ability to temperature program the Bendix instrument made it possible to separate the octadecene peaks from the tail of the solvent peak to a much greater degree than was possible isothermally. As a result, octadecene could now be detected at concentrations of 2 nanograms per microliter compared to 10 nanograms per microliter previously.

Reflux Method Without Hexane - The most successful

TABLE VI

REFLUX METHOD REPRODUCIBILITY USING HYDROXIDE FOR THE BASE. REACTION CONDITIONS WERE 70° C FOR 120 MINUTES

REACTION CONTENTS	RESULTS olefin yield (units of peak height x attenuation)
2ml 8N KOH	1. 46 x 20
1ml 500ppm Drug	2. 48 x 20
	3. 45 x 20
	4. 52 x 20
	5. 46 x 20

results of all were obtained when the reflux reaction was run with the hexane deleted from the system. Results are illustrated in Table I on page 22. The 100ppm reaction was run with hexane. The 10 and 1ppm reactions were run without hexane. In a departure from the practice described under Calculations, the 1ppm reaction had 2 grams of liver present instead of 200 milligrams. This was done to keep the amount of octadecene generated well within easily measurable bounds. The chromatographic background increased about threefold when this was done. See Figure IV on page 42. If the amount of liver was increased another tenfold to simulate a drug level of 0.1ppm, the background would obliterate the octadecene peak with present column conditions.

Some of the substances which are responsible for the large background may be successfully removed from the hexane containing the reaction products before it is concentrated by acidic or basic aqueous washing. The octadecene peaks may also be better resolved from the background by the use of a column of greater length and new temperature program parameters.

In conclusion, I feel that the liver background problem is one that can be overcome. In the interim, preliminary approval for field testing of the drug with this technique should be obtainable with final approval at 0.1ppm being very likely.

It must be pointed out that the absolute yields of octodecene mentioned herein must be interpreted with the

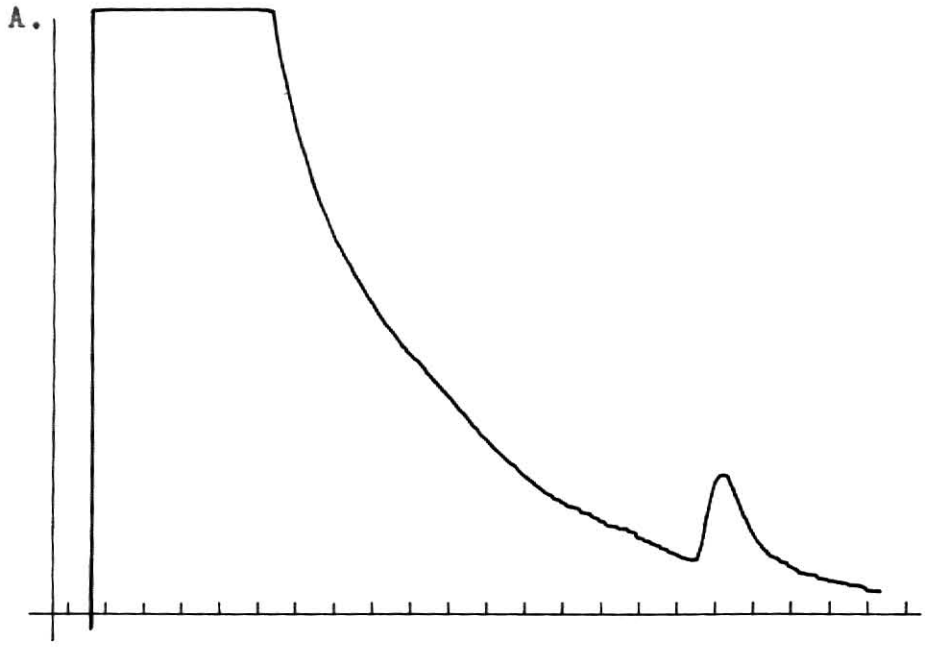
FIGURE IV

EXAMPLE CHROMATOGRAMS

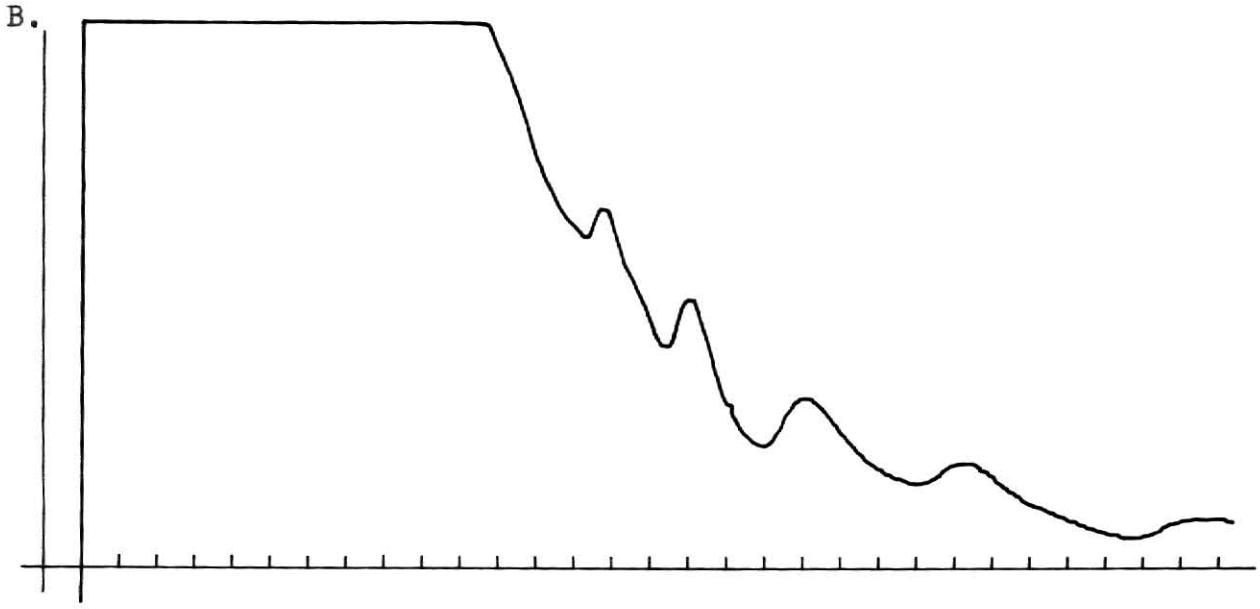
A. 5 MICROLITERS OF OCTADECENE STANDARD 8 NANOGRAMS PER MICROLITER

B. 5 MICROLITERS OF REACTION PRODUCTS 10PPM, 200MG LIVER PRESENT IN REACTION

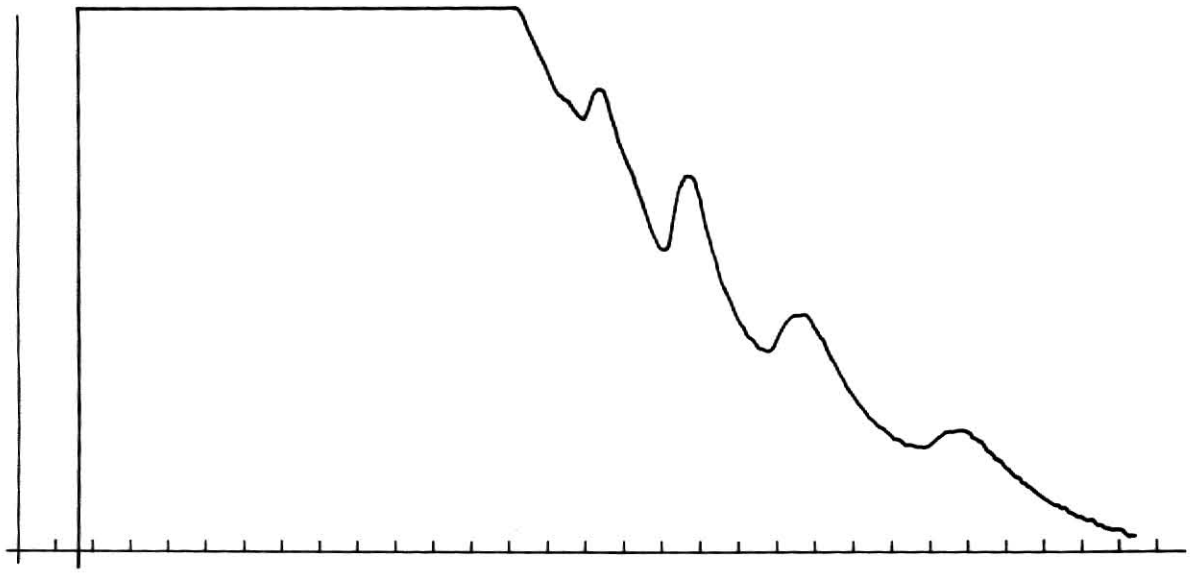
C. 5 MICROLITERS OF REACTION PRODUCTS 1PPM 2G LIVER PRESENT IN REACTION


A.

B.



c.



knowledge that the true absolute composition of the drug is not known. From conversation with Link at Ashland Chemical (18) it is quite apparent that the absolute content of 18 carbon alkyl moieties in the product is not well known.

Nevertheless, octadecene yields from the Hoffmann reaction can be used to detect TA-100 levels in beef liver of 1ppm and, as stated above, 0.1ppm levels are not expected to be impossible.

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AN ANALYTICAL METHOD FOR THE DETERMINATION OF A DIALKYL DIMETHYL QUATERNARY AMMONIUM CHLORIDE IN BEEF LIVER TISSUE

by

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B.S., Clemson University, 1971

AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

Arosurf TA-100 (16) has been developed by Meyer et al. (7) at the Department of Dairy and Poultry Science for the purpose of preventing grain bloat in beef and dairy cattle. In order for the drug to become commercially available, the Food and Drug Administration has stipulated (10) that the drug must ultimately be determinable in beef tissue at levels of 0.1ppm.

Liver was selected as the experimental tissue in which to develop an assay due to its function as the detoxification organ in the body.

The drug is a dialkyl dimethyl quaternary ammonium salt. Two analytical approaches were investigated: 1. A Hoffmann elimination reaction (11) was run in the hopes that the resultant olefin would provide a means of indirectly measuring how much quaternary amine was in the tissue. Gas liquid chromatography was used to measure how much olefin had been generated and 2. An attempt was made to extract the drug away from the tissue with an anionic dye molecule. An englomerate salt (15) would be formed. Once formed, it would be expected to extract into a nonpolar organic solvent. The amount of the amine dye englomerate extracted could then be measured colorimetrically.

The dye extraction method worked without liver present

however, apparently the liver bound to the drug more strongly than the dye. No extraction of amine dye conglomerate was demonstrated with liver drug samples.

The elimination reaction was found to proceed successfully at boiling water temperatures and reaction times of approximately ten hours in large test tubes fitted with cold water condensers. The technique has been successfully performed on samples at the 1ppm level. Reasons are given for the expectation of 0.1ppm assays to be achievable.