A SEMI-MICRO DETERMINATION FOR PLANT STEROLS

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A THESIS

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I have appreciated the helpful assistance
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I. INTRODUCTION

The increasing interest in the use of phytosterols as precursors for the synthesis of hormones and antirachitic vitamins as well as their expanding use in other commercial fields, seemed to indicate that a study of the development of these sterols in plants would be profitable. In order to make such a study the need for a method applicable to the estimation of sterols in general, to include both saturated and unsaturated, was very evident.

A survey of the literature revealed that there was no such method available. The gravimetric methods lacking the required sensitivity and the colorimetric methods lacking in their general applicability.

A determination of this type must meet two primary requirements. Firstly, the sterols must be separated from contaminating materials on a semi-micro basis and, secondly, the entire quantity of sterols separated, without regard for minor changes in structure, must be accurately estimated.

II. HISTORICAL

Windaus (1), in 1909, showed that cholesterol formed a complex with digitonin which was insoluble in 95% ethanol. Since that time the isolation and purification of naturally occurring sterols has largely depended on this reaction. In all cases studied, whether of animal or plant origin, the complex appears to be formed from equimolecular amounts of digitonin and sterol. Every sterol, with the exception of calosterol from the juice of Calotropis gigantis (2), isolated from natural sources forms such a complex and they all have limited solubilities.

In a critical investigation of this procedure Schoenheimer and Dam (3) found that from 95% ethanol the precipitation was not absolutely quantitative but varied according to the excess digitonin present. In 1937, it was reported by Sperry (4) that Schoenheimer had overcome this weakness of the method by the use of 80% ethanol, in which medium the precipitation was quantitative regardless of the excess of digitonin present.

Many other precipitating mediums have been

proposed, such as acetone (5), alcohol and chloroform (6) and ether (7) etc. but the above mentioned 80% ethanol appears to be the most satisfactory.

More recently Sobel et al (8, 9) have developed a method of precipitating cholesterol by the use of pyridine and chlorosulfonic acid, resulting in the formation of an insoluble pyridinium sulfate. The value of this method for the precipitation of mixed sterols of plant origin has not as yet been investigated. Indeed, whether or not all sterols form an insoluble complex under the conditions outlined has not been ascertained.

Another possibility for the purification of the plant sterols lies in the field of chromatographic separation. To the present time the work in this field has been limited and is ably reviewed by Zechmeister (10). The main line of approach has been the isolation of one particular sterol from all others or the separation of several sterols from a purified mixture. Ladenberg(11) made use of the colored azobenzene-p-carboxylic esters of the sterols

and carried out their separation on alumina. However, Ott (12) states, "It is now conceded that the separability of sterols on alumina columns depends primarily upon differences between the number of double bonds in the molecule, and not upon the position of the double bonds or the structure of the side chain."

Wall and Kelley (13) varied this approach in that they adsorbed interfering substances on a magnesium oxide and Super-Cel column and obtained the partially purified sterols in the eluate. The chlorophylls and carotenoids, with the exception of the carotenes, were thus removed. From this partially purified eluate it was found possible to precipitate the sterols as the digitonides and to subsequently remove the other lipids by washing with ether.

Assuming the separation of the sterols from contaminating substances there still remains the problem of their quantitative estimation. For this purpose many methods have been tried with greater or lesser success.

A number of colorimetric reactions have been reported for the sterols which have been

used for preliminary identification and some for quantitative estimation. The Salkowski reaction (14) depends on the color formed when a chloroform solution is layered with concentrated sulfuric acid and variations of this reaction using an alcoholic solution of the sterol (15) and using the alcoholic sterol solution to which an aldehyde has been added (16, 17). The Rosenheim test (18) differs from these in that 90% trichloracetic acid solution is used with the free sterol or with a chloroform solution of the sterol. None of these reactions has been adopted for quantitative work.

In 1932, Bernoulli (19) determined cholesterol by measuring the red color which developed when a glacial acetic acid solution was heated with acetyl chloride and anhydrous zinc chloride. Thornton (20) made a critical examination of this reaction and found that there was a characteristic transmission maxima at 440 mu for various sterols but that the extinction coefficient was different for the various sterols. It is, therefore, apparent that the use of this method for the

determination of a mixture must be based on an exact knowledge of the ratio of the various sterols present.

The Liebermann - Burchard reaction (21, 22) resulting in the development of a blue color on the addition of acetic anhydride and sulfuric acid to solutions of sterols has been the most widely investigated of the color reactions. In 1934, Schoenheimer and Sperry (7) developed this reaction to the point where it has been almost solely used for the estimation of cholesterol in animal tissues and fluids. Attempts have been made to adapt this reaction to plant sterols (13, 23) but again it has been found that different sterols develop different colors. Both the above authors used an empirical approach to the problem by isolating macro amounts of the sterol mixture from a particular plant species and using this isolated material for the preparation of a working curve. However, it was still necessary to make the assumption that the ratio of the amounts of the various sterols present was the same in all cases.

None of the color reactions reported is

applicable to saturated sterols and therefore they cannot be used for the quantitative estimation of this fraction.

Rappaport and Klapholz (24) precipitated the cholesterol by use of a standard solution of digitonin and then determined the excess of digitonin present by hydrolysing with sulfuric acid and quantitatively estimating the reducing sugars liberated.

Windaus (1), in his original method, isolated the cholesterol digitonide, dried and weighed it. The amount of cholesterol present was then determined by multiplying by an appropriate factor, depending on the relative molecular weights of cholesterol and digitonin, the factor for cholesterol being 0.242. This procedure has been adapted for use with plant sterols by Wall and Kelley (13). The factor used in this case was 0.253 based on the average molecular weight of the various plant sterols. By such a means the saturated and unsaturated sterols can be estimated together, then the saturated sterols can be determined through the removal of the unsaturated ones by the method

of Anderson and Nabenhauer (13, 25). By difference the unsaturated sterols can be estimated.

Muhlbock and his coworkers (26) estimated cholesterol by isolating the digitonide, redissolving it in a mixture of methyl and ethyl alcohol and reprecipitating it by the addition of water and recording the turbidity of the resulting mixture.

Szent-Gyorgyi (27), in 1923, and Okey (28), in 1930, developed methods for the quantitative estimation of cholesterol which depended on the dichromate oxidation of the digitonide followed by an iodometric titration of the excess dichromate. These methods approached the required sensitivity but were time consuming and somewhat lacking in precision. Later attempts by various workers (6, 29, 30, 31) resulted in only moderate improvement. MacLachlan (32) directly extrapolated Boyd's oxidative method to germinating soybeans, but estimated that the results were probably about 10% low.

III. EXPERIMENTAL

It was thought that an investigation of four different types of procedures might result in an adequate method.

1. Some reaction involving the hydroxyl group, which is common to all the sterols, as the reactive centre; e.g.- preparation of an ester followed by hydrolysis and determination of the liberated acid.
2. The use of nephelometry to determine the rate and density of the precipitation of the digitonides.
3. Hydrolysis of the digitonide followed by the estimation of the liberated reducing sugars.
4. Total oxidation of the digitonide.

Preliminary studies were made on these methods in the hope that one or more might yield encouraging results.

Throughout the work the following sterols were used as standard preparations:-

1. Cholesterol - Difco Laboratories Cholesterol; M.P. 148-149°C used as purchased.
2. Stigmasterol - prepared from crude soybean

sterols and recrystallized a total of fourteen times as the acetate and free sterol; M.P. 166-167°C.

3. Sitosterol - Nutritional Biochemicals Corporation Sitosterol, recrystallized once from absolute ethanol; M.P. 136.5-137.5°C.

4. Corn sterols - extracted from a sample of "soap stock", obtained from the Canada Starch Co., with hexane, evaporating the extract to dryness and recrystallizing twice from aqueous alcohol; M.P. 137.5-139°C.

A. Preparation of Esters

Acetylation - Because of the ease of removing the excess reagent, acetyl chloride was used throughout as the acetylating reagent. The isomerism resulting from the liberated hydrochloric acid was disregarded because only the total sterols were to be determined. No attempt to identify individual sterols was anticipated.

The standard methods of determining acetyl groups were tried but it was found that creeping of the reagents occurred in the usual apparatus with acid creeping mechanically into the receiving flask during distillation. Also the direct saponification of the ester with alcoholic alkali followed by titration of the excess alkali was unsatisfactory. In the latter case no satisfactory end point could be reached because there appeared to be a gradual, erratic release of alkali over a period of several days.

The most satisfactory results using this method were obtained as follows:-

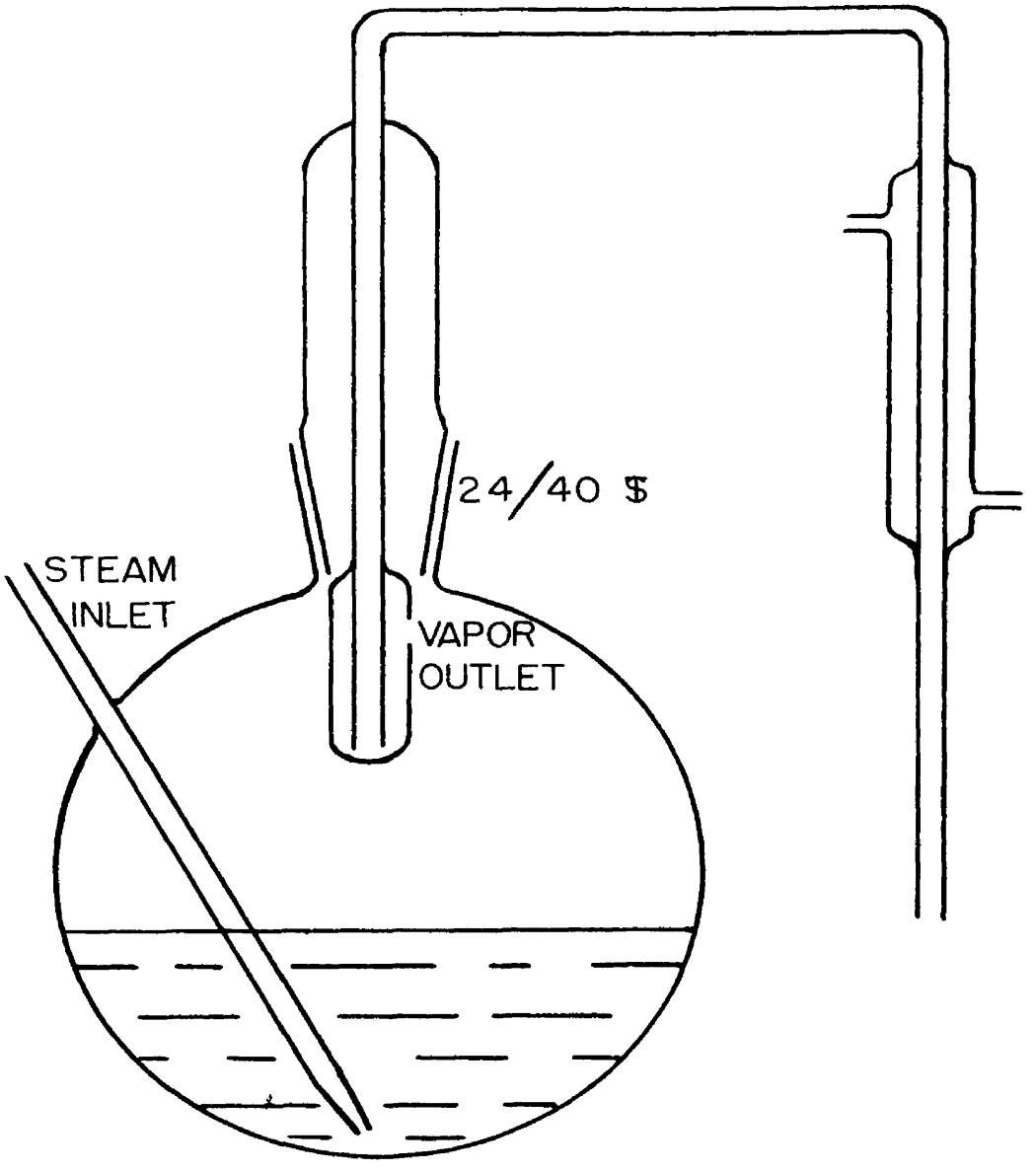
Samples containing zero, twenty-five and fifty mgm. of cholesterol were weighed into short necked micro Kjeldahl flasks fitted with 24/40

standard taper necks. Two ml. of acetyl chloride was added to each sample and they were then refluxed for one hour. The excess acetyl chloride was removed by means of a water aspirator at room temperature followed by drying in a vacuum over at 80°C for twenty-four hours.

The acetylated samples were then refluxed for one hour with fifty percent sulfuric acid and the liberated acetic acid was distilled, using a specially designed distillation head, Figure 1. This equipment was designed to prevent the creeping of the sulfuric acid which occurs in the standard apparatus. Approximately fifty ml. of distillate was collected and it was immediately titrated with 0.5 N sodium hydroxide using phenol red as an internal indicator.

Typical results obtained by this method are illustrated in Table 1.

As can be seen from these figures the results are variable and are not adequately reproducible even when using relatively large samples. It was not believed feasible to improve both the precision and sensitivity of such a method for the required use.



APPARATUS FOR DISTILLING HAc FOR TITRATION

FIG. 1

TABLE 1

CHOLESTEROL DETERMINED BY ACETYLATION AND TITRATION OF LIBERATED ACETIC ACID

Mgm. Cholesterol Present	Ml. .05 N NaOH	Mg. Cholesterol Found
0	0.06	0.0
25	1.26	23.2
50	2.28	42.8
0	0.06	0.0
25	1.28	23.3
50	2.81	51.9
0	0.04	0.0
25	0.58	11.2
50	1.40	27.1

It was thought that if a highly colored ester could be prepared and isolated that a colorimetric method might be developed which did not depend on the relative structures of the various sterols. However, attempts to prepare and purify semi-micro amounts of the p-phenylazobenzoyl esters quantitatively according to Ladenberg's procedure (11) failed.

Attempts were also made to prepare the glycyl ester of the sterols with a view to determining the nitrogen present in the ester by means of a micro Kjeldahl technique. These attempts were unsuccessful.

B. Use of the Nephelometer

Preliminary investigation with cholesterol indicated that the flocculation of the digitonide occurred fairly rapidly and that this flocculation greatly affected the results obtained. Search for a stabilizer resulted in the use of a gum ghatti solution for this purpose. During the preliminary investigation using purified solutions of sterols the turbidity was read directly on the precipitating digitonide.

Experimental

Preparation of stock solutions

Digitonin solution:- 600 mgm. of digitonin (Merck) was dissolved in 200 ml. of 95% ethanol by heating, 800 ml. of distilled water was added; to this mixture 50 ml. of 2% gum ghatti solution was added. The whole solution was thoroughly mixed.

Sterol solutions:- A solution of cholesterol was prepared in 95% ethanol so that 2 ml. would contain 0.1, 0.2, 0.3, 0.4, 0.5 mgm. of the cholesterol. Similar solutions of ergosterol and stigmasterol were prepared.

Procedure

Two ml. of the sterol solution was pipetted into a nephelometer cuvette and five ml. of digitonin solution added. The mixture was shaken vigorously for thirty seconds and then allowed to stand until read. All readings were made on a Coleman Photo-Nephelometer.

Results

Preliminary studies without the addition of gum ghatti resulted in a rapid change in nephelometer readings as shown in Table 2. The addition of the gum ghatti resulted in a much more stable condition over fairly long time intervals as is illustrated in Table 3.

From the results of Table 3 each of the following samples was allowed to stand one hour prior to reading. Using this time the values for cholesterol, ergosterol and stigmasterol are to be found in Table 4 and represented graphically in Figure 2, using 0.1 mg. of cholesterol as an instrumental standard. From Table 4 and Figure 2 it can be readily seen that the rate, volume and probably the particle size of the precipitates vary over a wide range.

TABLE 2

CHANGE IN NEPHELOMETER READINGS WITH TIME

NO GUM GHATTI

Mg. Cholesterol	Nephelometer Readings	
	1 Min.	15 Min.
0.1	1.85	2.30
0.2	4.18	4.45
0.4	7.79	8.38
0.5	9.50	10.24
0.1*	2.00*	2.15
0.2	3.90	4.18
0.4	7.85	8.50
0.5	9.70	10.50

* Used to standardize instrument.

TABLE 3

CHANGE IN NEPHELOMETER READINGS WITH TIME

GUM GHATTI ADDED

Mg. Cholesterol	Nephelometer Readings					
	Time in Minutes					
	5	20	60	90	600	2880
0.1	1.45	1.79	1.85	1.90	1.80	1.95
0.2	3.50	3.77	3.85	3.85	3.65	3.75
0.3	5.65	5.85	5.85	5.80	5.70	6.00
0.4	7.75	7.96	7.85	7.90	7.50	8.95
0.5*	10.00	10.00	10.00	10.00	10.00	10.00

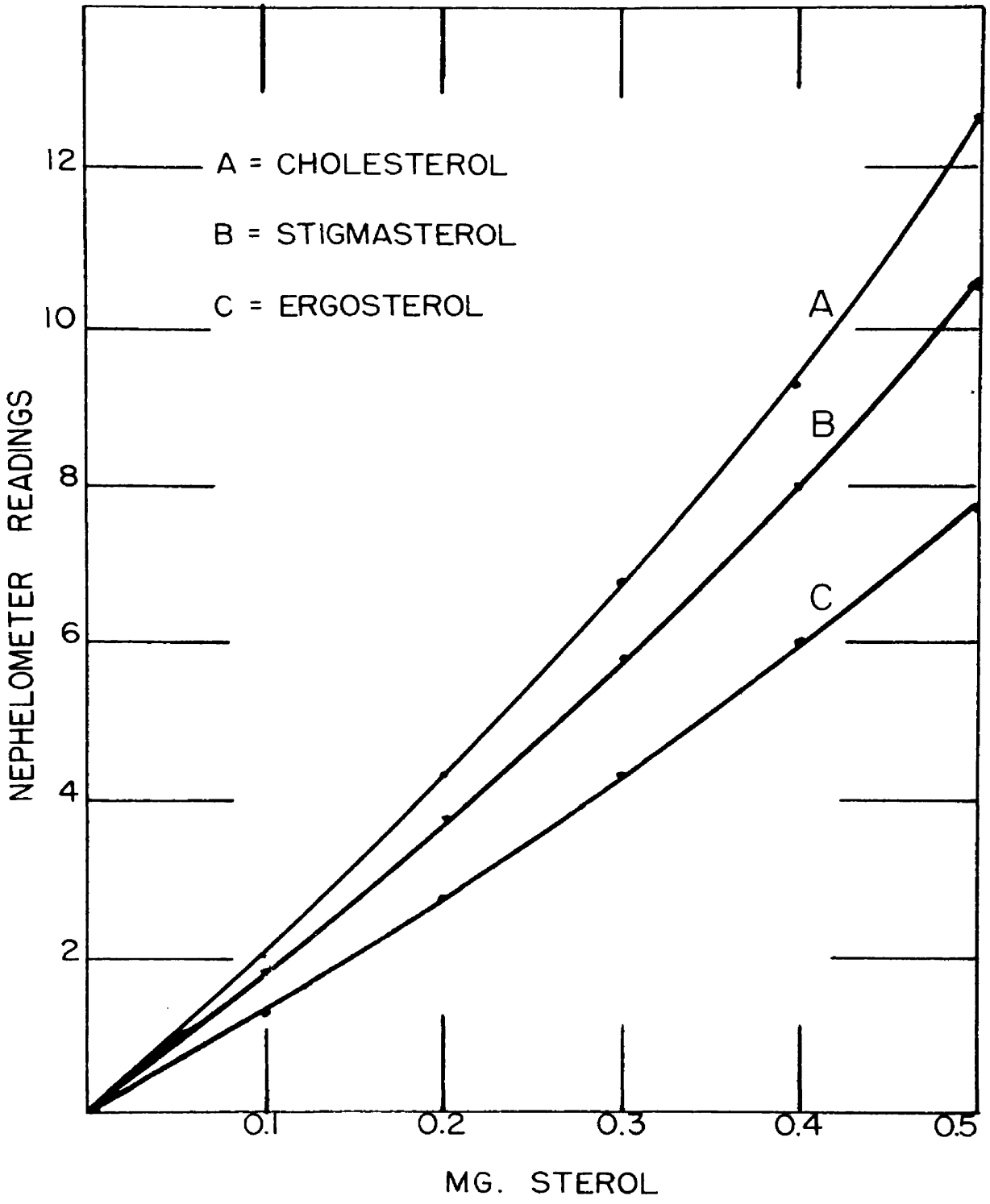
* Used to standardize instrument.

TABLE 4

NEPHELOMETER READINGS FOR CHOLESTEROL, ERGOSTEROL AND STIGMASTEROL

Mgm. Sterol	Nephelometer Readings		
	Cholesterol	Ergosterol	Stigmasterol
0.1*	2.00*	1.28	1.85
0.2	4.32	2.78	3.81
0.3	6.72	4.30	5.88
0.4	9.24	5.92	8.05
0.5	12.54	7.50	10.46

* Used to standardize the instrument for all readings.



MEASUREMENT OF TURBIDITY USING 0.1 MG. OF CHOLESTEROL AS STANDARD

FIG. 2

In order to obviate the necessity of using cholesterol as a standard for the other sterols these series were repeated using the 0.1 mgm. level of each sterol as the instrumental standard for that particular series. These results are shown in Table 5 and Figure 3.

It becomes evident from these results that such a determination might be entirely feasible for solutions of pure sterols but that if a mixture of sterols is present that an intimate knowledge of the individual sterols present would be required.

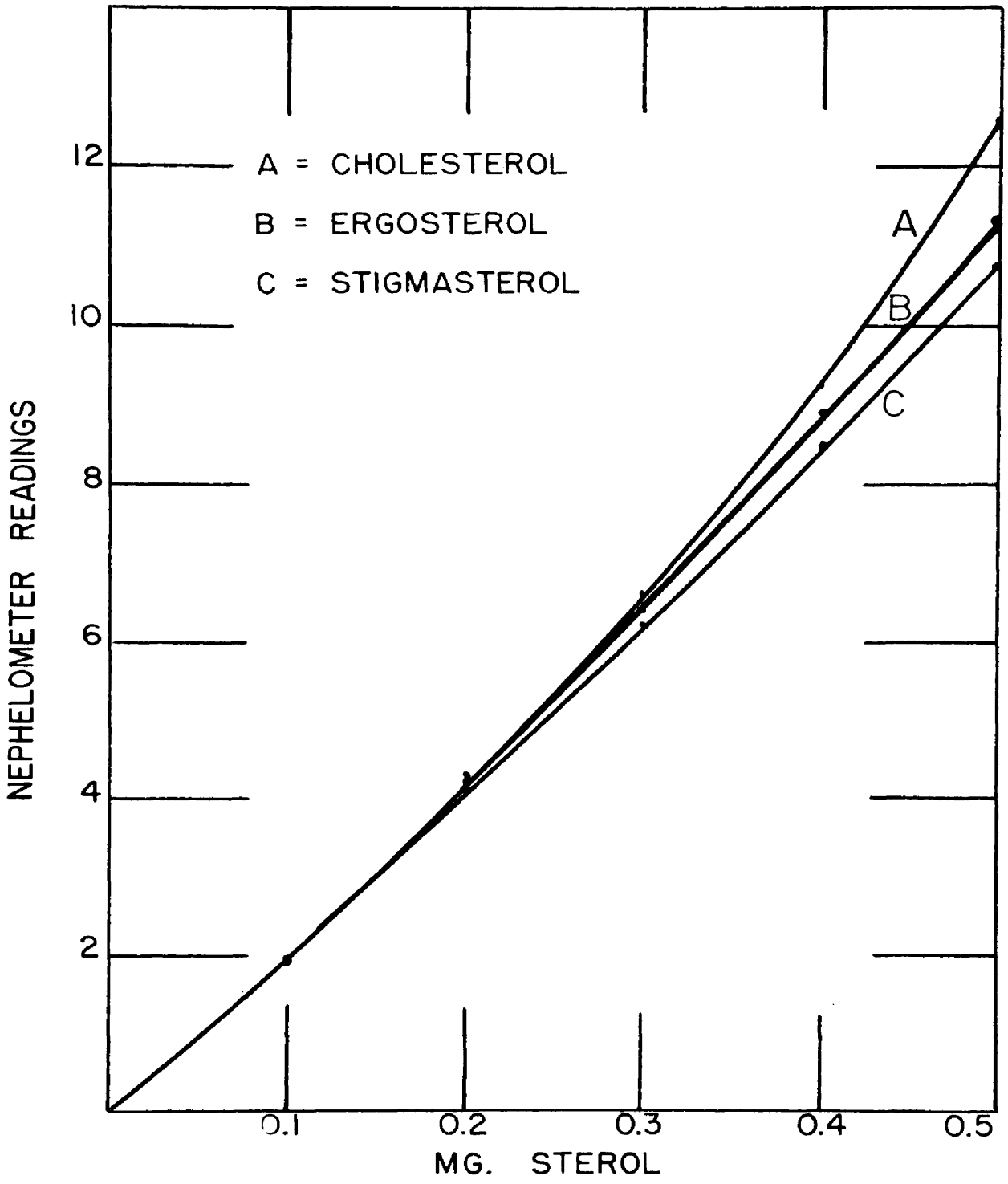
It would appear from these results, however, that the rate and density of precipitation increases with the decreasing of the number of double bonds.

TABLE 5

NEPHELOMETER READINGS FOR CHOLESTEROL, ERGOSTEROL AND STIGMASTEROL

Mgm. Sterol	Nephelometer Readings		
	Cholesterol	Ergosterol	Stigmasterol
0.1*	2.00*	2.00*	2.00*
0.2	4.32	4.26	4.11
0.3	6.72	6.65	6.33
0.4	9.24	9.06	8.52
0.5	12.54	11.39	10.82

* Instrument standardized by 0.1 mg. level of each sterol for its own series.



MEASUREMENT OF TURBIDITY USING 0.1 MG. OF EACH STEROL AS ITS OWN STANDARD

FIG. 3

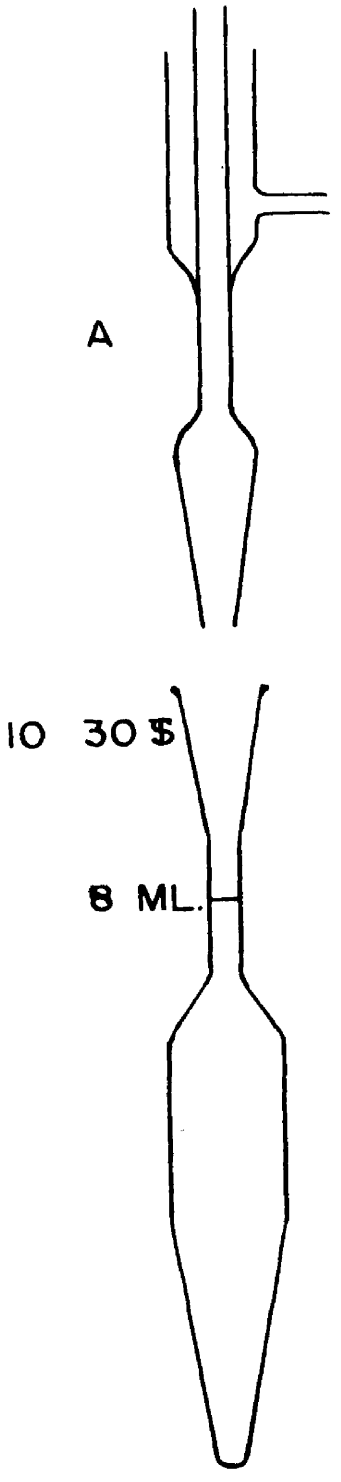
C. Hydrolysis of the Digitonide Followed by the Determination of the Liberated Reducing Sugars

Rappaport and Klapholz (24) precipitated cholesterol, hydrolysed the excess digitonin in the mother-liquor and then quantitatively determined the reducing sugars liberated. It was thought that the mother-liquor from the precipitation of the sterols from partially purified plant extracts might contain a variable amount of other substances capable of simulating sugars in their reducing ability. This is especially true when the excess digitonin in the mother-liquor is subjected to hydrolysis in the presence of a strong acid (4.0 N sulfuric acid) for a period of two hours. In order to overcome this difficulty the digitonide was isolated, purified, dried and then subjected to hydrolysis.

Preliminary investigations indicated that it was impossible to transfer the precipitate from a centrifuge tube after purification to a flask for hydrolysis. Due to this fact a centrifuge tube was designed which permitted centrifuging, washing, hydrolysis and dilution to standard volume to be accomplished without

transfer. This tube is shown in Figure 4. It was then found that during the active boiling required for the hydrolysis the precipitate was carried up onto the walls of the tube and stuck there. In this manner it was removed from the reaction mixture and did not undergo hydrolysis. This was overcome by adding a few drops of n-amyl alcohol to the tube. As the alcohol recondensed during hydrolysis it washed down the sides of the tube and also prevented excessive frothing. Another advantage found was that the sterol and the aglucone of the digitonin were soluble in the alcohol layer whereas the sugars liberated were soluble in the water layer. Thus the completion of hydrolysis could be determined by the disappearance of the precipitate.

The biggest difficulty was found to be the control of the rate of boiling during hydrolysis. Many anti-bump agents, including boiling tubes, carborundum, teflon, beads, small pieces of fritted glass, etc., were tried without too much success. The boiling tube was perhaps the most successful and was used for most of this work.



TUBE USED FOR CENTRIFUGING, WASHING, HYDROLYSING, AND DILUTION TO VOLUME

FIG. 4

In addition to an adequate lack of control these boiling tubes were found hard to rinse free of all solution.

Preparation of Reagents

Digitonin solution:- one gm. of digitonin (Merck) was dissolved in one litre of water and then reduced in volume to 500 ml.

Cholesterol solutions:- solutions of cholesterol in 95% ethanol were prepared, containing 0.25, 0.50, 0.75, 1.00, 1.25 mgm. per five ml.

4 N Sulfuric acid.

4 N Sodium hydroxide.

n-Amyl alcohol (redistilled).

Alkaline copper and phosphomolybdic acid solutions were prepared according to Folin and Wu (33).

Experimental

Five ml. aliquots of each of the solutions of cholesterol were pipetted into individual centrifuge tubes of special design and 2.5 ml. of digitonin solution added. The contents were mixed and heated to coagulate the precipitate and left overnight to complete the precipitation.

The following day the tubes were centrifuged and the supernatant liquid decanted. The precipitates were washed by centrifugation and decantation. After washing, one ml. of 4 N sulfuric acid was added and the tubes refluxed for four hours; Figure 4. The contents were neutralized by the addition of one ml. of 4 N sodium hydroxide and diluted to eight ml. The amyl alcohol layer was removed with a fine capillary and suction and two ml. aliquots were pipetted into Folin-Wu sugar tubes. Four ml. of the alkaline copper reagent was added and the tubes were then placed in a rapidly boiling water bath for twenty minutes. After heating the tubes were cooled in running water without agitation and four ml. of the phosphomolybdic acid solution was added to each. The contents were mixed, allowed to stand until all evolution of gas ceased, diluted to 25 ml. and mixed thoroughly. All color readings were made in a Coleman Universal spectrophotometer at 420 mu.

The color developed by this method was found to be unstable with time as is illustrated in Table 6.

TABLE 6

CHANGE OF COLOR WITH TIME USING FOLIN-WU REAGENT

Mg. Cholesterol	Spectrophotometer Readings 420 mu				
	0 Time	30 min.	150 min.	5 hrs.	21 hrs.
0.00	100	100	100	100	100
0.25	75.5	77.5	82.0	84.0	81.0
0.50	56.0	57.5	64.0	69.0	69.0
0.75	47.5	47.5	54.0	59.0	61.5
1.00	35.5	35.0	37.5	42.5	50.0
1.25	33.5	33.0	34.5	39.0	47.0

Because of this unstability of color developed by the Folin-Wu reagent, Nelson's (34) modification of the Somogyi reaction was investigated. The color developed with this reagent was much more stable and more concordant results were obtained. The color development in the high ranges of sterol concentration with this reagent, was too intense for practical use and a new series of cholesterol standards was prepared containing 0.1, 0.2, 0.3, 0.4, 0.5 mgm. of cholesterol per five ml. respectively.

Using these substitutions the results of duplicate series A and B shown in Table 7 were obtained.

Again the color developed seemed to be too intense for accuracy so the solutions were diluted to fifty ml. before reading and these results, duplicate series C and D, are shown in Table 8.

These results seemed to be of the desired accuracy and a working curve was prepared by averaging the results of these two series, Figure 5.

Solutions of stigmasterol containing

TABLE 7

COLOR DEVELOPMENT USING NELSON'S REAGENT

Mgm. Cholesterol	Spectrophotometer Readings 420 mu.	
	Series A	Series B
0.00	100	100
0.10	58.5	59.0
0.20	34.0	35.0
0.30	21.0	22.0
0.40	12.0	13.5
0.50	8.0	8.5

TABLE 8

COLOR DEVELOPMENT USING NELSON'S REAGENT DILUTION TO 50 ml.

Mgm. Cholesterol	Spectrophotometer Readings 420 mu.	
	Series C	Series D
0.00	100	100
0.10	78.5	80.0
0.20	61.0	63.0
0.30	49.0	49.0
0.40	38.0	39.0
0.50	31.0	32.0

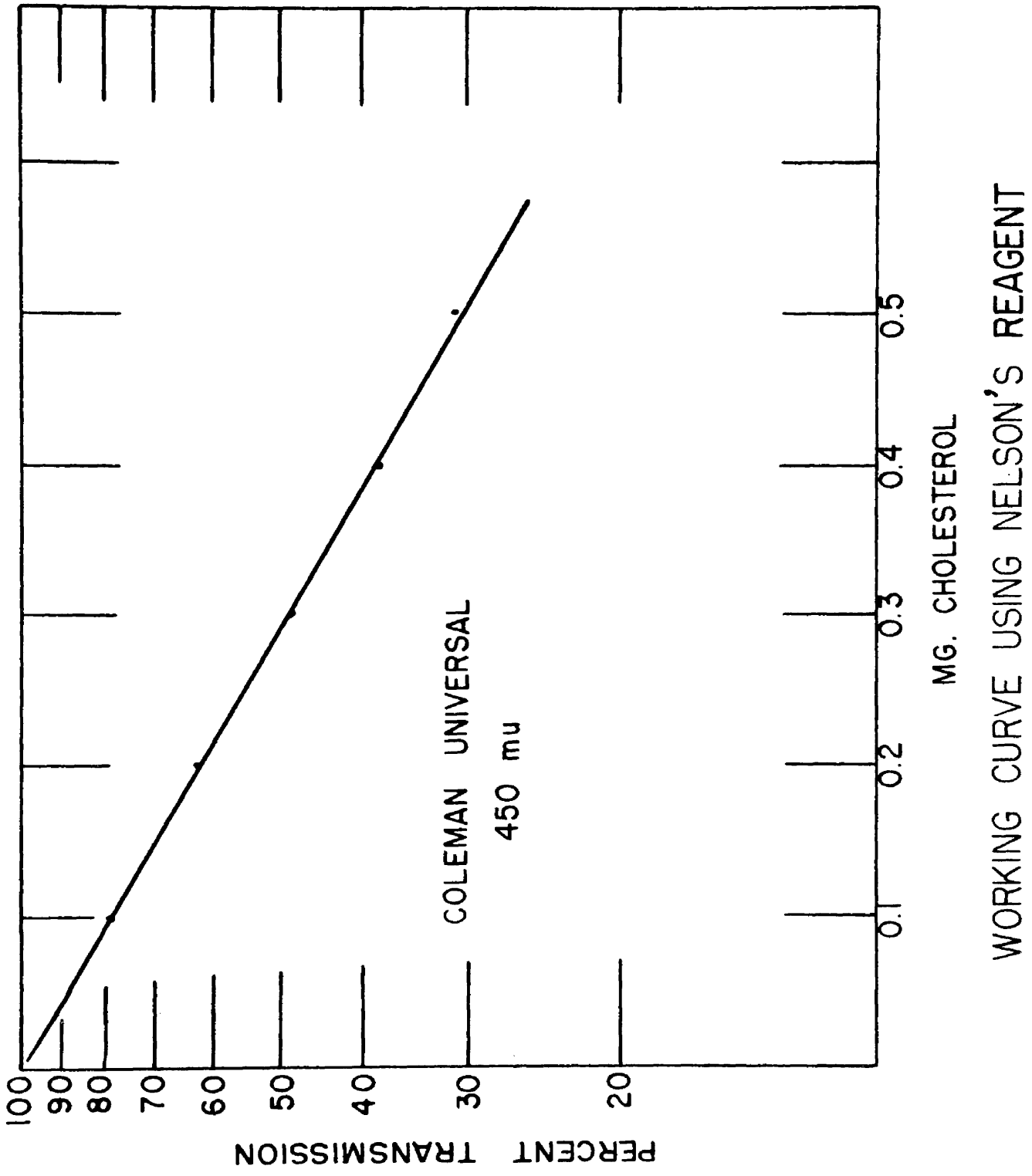

WORKING CURVE USING NELSON'S REAGENT
MG. CHOLESTEROL
0.1
0.2
0.3
0.4
0.5
COLEMAN UNIVERSAL
450 mu
PERCENT TRANSMISSION
100
90
80
70
60
50
40
30
20

FIG. 5

0.1, 0.2, 0.3, 0.4, 0.5 mgm. per 5 ml. were prepared and the sterol content was determined using the cholesterol working curve. Four replicates A, B, C and D of each level of the sterol were determined individually. The error was calculated to see whether or not reproducibility was being obtained, Table 9.

The results with stigmasterol showed some promise and so solutions of mixed soybean sterols, supplied by Prof. C. D. Ball, were prepared containing equivalent amounts to the previously used cholesterol and stigmasterol solutions. The results obtained with these solutions are shown in Table 10. Again four replications A, B, C and D were determined individually.

The results with soybean sterols (Table 10) appeared to be very low. However, if this mixture of sterols were to contain 20% impurities the percentage errors shown in Table 11 would have resulted.

Further series of determinations resulted in errors of approximately the same magnitude and it was found that while reasonable duplication

TABLE 9

STIGMASTEROL DETERMINED FROM CHOLESTEROL WORKING CURVE

Stig. Present	T 450 mu.	Stig. Found	Mg. Error	% Error
		Series A		
0.00	100	0.00	0	0
.10	77.5	.1075	+ .0075	+ 7.5
.20	59.0	.2220	+ .0220	+11.0
.30	49.0	.3010	+ .0010	+ 0.3
.40	39.0	.3980	- .0020	- 0.5
.50	32.0	.4820	- .0180	- 3.6
		Series B		
0.00	100	0.00	0	0
.10	76	.1175	+ .0175	+17.5
.20	59.0	.2220	+ .0220	+11.0
.30	46.5	.3350	+ .0350	+11.6
.40	37.5	.4150	+ .0150	+ 3.75
.50	33.0	.4750	- .0250	- 5.0

(Continued)

TABLE 9 (Cont'd)

STIGMASTEROL DETERMINED FROM CHOLESTEROL WORKING CURVE

Stig. Present	T 450 mu.	Stig. Found	Mg. Error	% Error
		Series C		
0.00	100	0.00	0	0
.10	79.5	.097	- .003	- 3.0
.20	62.0	.201	+ .001	+ 0.5
.30	49.0	.301	+ .001	+ 0.3
.40	40.0	.386	- .004	- 1.0
.50	35.0	.444	- .056	-11.2
		Series D		
0.00	100	0.00	0	0
.10	79.0	.100	.100	0
.20	60.5	.213	+ .013	+ 6.5
.30	50.0	.292	- .008	- 2.6
.40	40.0	.386	- .004	- 1.0
.50	35.0	.444	- .056	-11.2

TABLE 10

MIXED SOYBEAN STEROLS DETERMINED FROM CHOLESTEROL WORKING CURVE

Soy Sterol Present	T 450 mu.	Soy Sterol Found	Mg. Error	% Error
		Series A		
0.00	100	0.00	0	0
.10	82.0	.083	- .017	- 17.0
.20	68.0	.162	- .038	- 19.0
.30	56.5	.240	- .060	- 20.0
.40	48.5	.305	- .095	- 23.25
.50	42.5	.360	- .140	- 28.0
		Series B		
0.00	100	0.00	0	0
.10	83.0	.078	- .022	- 22.0
.20	67.5	.165	- .035	- 17.5
.30	55.0	.252	- .048	- 16.0
.40	48.0	.310	- .090	- 22.5
.50	43.0	.356	- .144	- 28.8

(Continued)

TABLE 10 (Cont'd)

MIXED SOYBEAN STEROLS DETERMINED FROM CHOLESTEROL WORKING CURVE

Soy Sterol Present	T 450 mu.	Soy Sterol Found	Mg. Error	% Error
		Series C		
0.00	100	0.00	0	0
.10	85.0	.068	- .032	- 32.0
.20	65.5	.178	- .022	- 11.0
.30	57.0	.237	- .063	- 21.0
.40	50.0	.294	- .106	- 26.5
.50	39.0	.397	- .103	- 20.6
		Series D		
0.00	100	0.00	0	0
.10	74.5	.124	+ .024	+ 24
.20	68.5	.159	- .041	- 20.5
.30	57.0	.178	- .022	- 11.0
.40	46.0	.327	- .073	- 18.25
.50	40.0	.386	- .114	- 22.8

TABLE 11

PERCENT ERROR FOR SOYBEAN STEROLS ASSUMING 20% CONTAMINATION

Series A	Series B	Series C	Series D
+ 3.00	- 2.00	-12.00	+44
+ 1.00	+ 2.50	+ 9.00	- 0.50
0.00	+ 4.00	- 1.00	+ 9.00
- 3.25	- 2.50	- 6.50	+ 1.25
- 8.00	- 8.80	- 0.60	+ 2.80

could be expected on samples carried through side by side that duplication from day to day was much more uncertain. In order to investigate this point several series of known amounts of cholesterol were run on successive days and the results plotted in Figure 6.

In view of this variation, which appears to be in the hydrolysis and which up to the present has not been controlled, this method was temporarily abandoned and investigation of the oxidation of the digitonide begun.

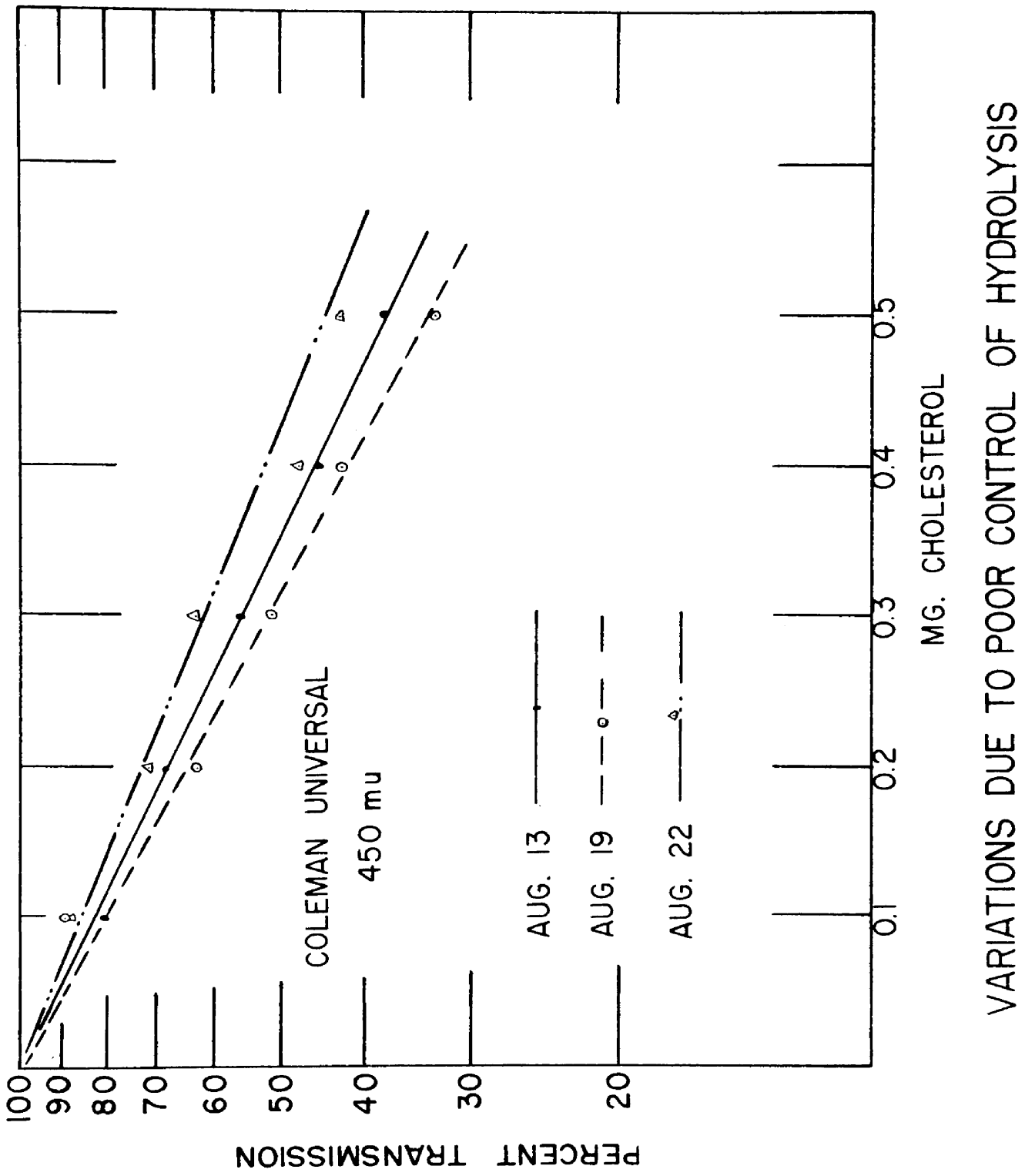

VARIATIONS DUE TO POOR CONTROL OF HYDROLYSIS
MG. CHOLESTEROL
0.1
0.2
0.3
0.4
0.5
AUG. 13
AUG. 19
AUG. 22
COLEMAN UNIVERSAL
450 mu
PERCENT TRANSMISSION
100
90
80
70
60
50
40
30
20

FIG. 6

D. Oxidation of the Digitonide

Reagents (all reagents analytical grade)

80% Ethanol - 95% ethanol diluted with distilled water.

1% Digitonin solution - 1 gm. of digitonin (Merck) dissolved in 100 ml. of 80% ethanol with warming. No variability was noted in results after two months usage if stored in a glass stoppered flask.

Absolute ethanol - Canadian Industries Limited absolute spirits.

Potassium dichromate - 29.422 gms. of dried potassium dichromate dissolved in 100 ml. of distilled water and made up to 1 litre with concentrated sulfuric acid. The use of Nicloux's reagent is not necessary.

Ferrous ammonium sulfate - 39.2 gms. ferrous ammonium sulfate dissolved in 1 M sulfuric acid and made up to 1 litre with 1 M sulfuric acid.

Procedure

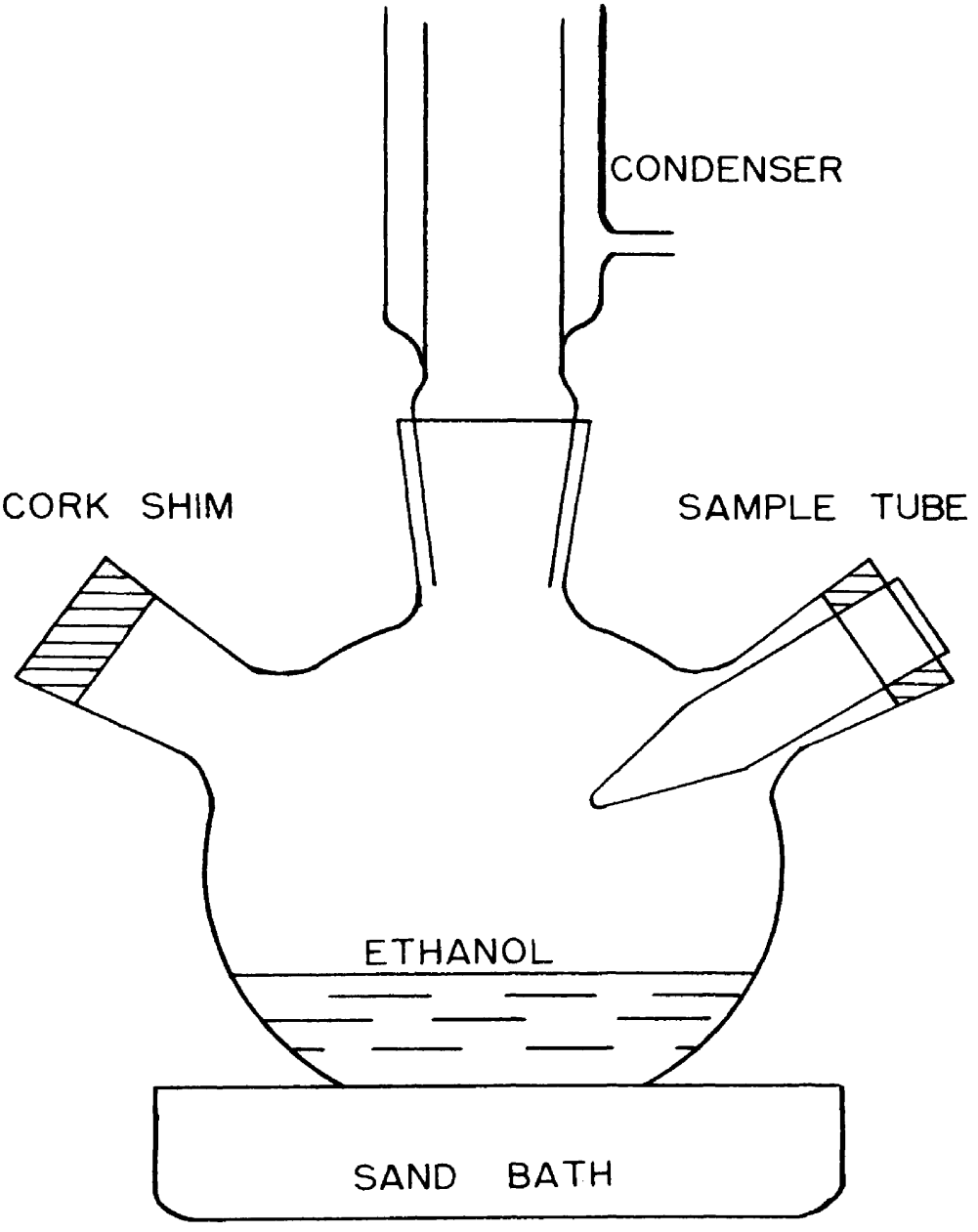
The extraction of the plant material and its

subsequent partial purification for precipitation was done by the use of Wall and Kelley's procedure (13), substituting Canadian Mineral Spirits "Hexane", B.P. 150-158°F., for Skellysolve B.

Following the adsorption on magnesium oxide and Super-Cel of a sample of plant extract equivalent to 0.25 - 1.0 gm. of air dry plant material (depending on the sterol content optimum amount of sterol about 0.25 - 1.0 mg. sterol although amounts ranging from 0.15 - 2.0 mg. can be determined under these conditions) the eluate is evaporated just to dryness on a well ventilated steam bath. The residue is cooled, taken up with hexane and filtered directly into a 15 ml. centrifuge tube. For routine analysis it is advantageous to adsorb one larger sample, evaporate to dryness, filter into a 25 ml. volumetric flask, make up to volume, mix and take aliquots of the required volume. The solvent is then evaporated once again to dryness. In order to obviate the necessity of using boiling tubes this evaporation was carried out in the vapors of rapidly boiling 95% ethanol. Under these

conditions evaporation is rapid and no active boiling occurs. If all the hexane is not removed by this treatment the tubes are transferred to a steam bath to remove the final traces of hexane. It is essential that all the solvent be removed at this time, or it will interfere with the subsequent precipitation.

Precipitation of the digitonides is accomplished by suspending the tubes in the vapors of boiling ethanol as shown in Figure 7. Five ml. of absolute ethanol are added, taking care to wash down thoroughly the sides of the tubes. Nothing further is added until all the residue has dissolved. This is followed by the addition of 2 ml. of digitonin solution and 1.25 ml. of distilled water. The contents are thoroughly mixed by means of a glass thread which is in turn rinsed with 80% ethanol. The tubes are heated for about a minute longer and then placed in pint preserving jars with a screw top and sealed (7). The jars have about one quarter inch of 80% ethanol in the bottom. In this manner precipitation occurs overnight with a minimum of evaporation.



REFLUX FOR EVAPORATION OF HEXANE AND PRECIPITATION OF DIGITONIDES

FIG. 7

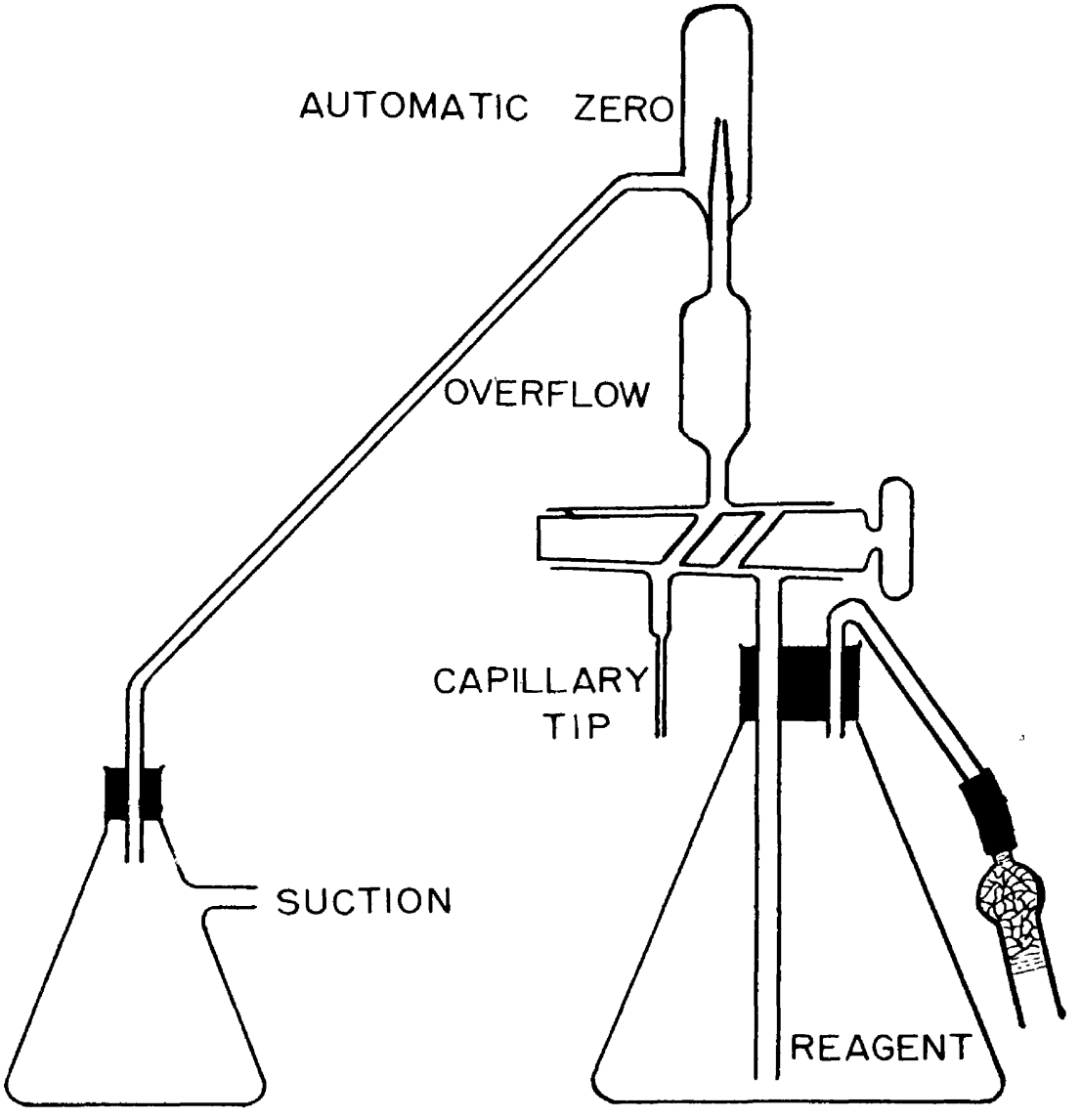
After overnight precipitation, any precipitate sticking to the walls of the tube is loosened by rubbing with a glass thread and rinsing down with 80% ethanol. The tubes are then centrifuged at 3000+ r.p.m. for one-half hour and the supernatant liquid is removed by means of a capillary tube and gentle suction. Decantation is not satisfactory with all samples because the precipitate does not pack sufficiently, this is particularly true of the smaller samples. In some cases a floating gelatinous precipitate is present which may range from a faint cloudiness to large discreet particles. This is removed with the supernatant liquid and does not appear to contain any of the sterols. The precipitate is washed once with 80% ethanol, using about 3 ml. and thoroughly mixing the precipitate with a glass thread. If the concentration of ethanol is correct the thread may be readily rinsed free of precipitate with 80% ethanol, if the ethanol concentration is much less than 80% this is impossible. The tubes are again centrifuged and the supernatant liquid removed as before. This washing removes the

excess digitonin. The precipitate is then washed twice, at least, with ethyl ether using the above technique, in order to remove other lipids.

After the final removal of the ether, the tubes are allowed to stand for about two hours, in practice they are allowed to stand overnight, in a covered beaker at room temperature. Then drying is completed by placing them in an oven at 90-95°C. for about one hour. By this means all danger of spattering and loss of precipitate is avoided.

The tubes are then cooled and a measured quantity of sulfuric acid - potassium dichromate reagent is added. The exact amount of reagent added is immaterial providing that there is an adequate excess (25%) and that the amount can be accurately duplicated. In practice, the authors have been using just over 4 ml. dispensed from a specially designed pipette (Figure 8) modified from MacDougall and Biggs (35), which has been very satisfactory as shown by Table 12.

Immediately after the reagent has been added, the tubes are suspended in a steam bath to a depth approximately one quarter inch above



AUTOMATIC PIPETTE FOR $K_2Cr_2O_7 - H_2SO_4$ REAGENT

FIG. 8

TABLE 12

STABILITY OF $K_2CR_2O_7$ - H_2SO_4 REAGENT AND REPRODUCIBILITY OF MEASUREMENT WITH PIPETTE

Sample No.	Date	Ml. Fe++ Sol'n
A	May 21	23.15
B	" 22	23.15
C	" 25	23.15
D	" 28	23.15
E	" 29	23.20
F	" 30	23.15
G	June 8	23.15

the surface of the reagent. This is accomplished by slipping pieces of rubber tubing over the tubes to support them on the steam bath cover.

The tubes are left stationary for three and one-half hours after which time they are inspected and gently rotated in a sloping position. If any precipitate is clinging to the sides of the tube it is rinsed down by gentle agitation. Heating is then continued for another one-half hour.

The contents of the tubes are then quantitatively rinsed into beakers using 40-50 ml. of distilled water, 1 drop of ortho-phenanthroline ferrous complex indicator (36) is added and the excess dichromate is then titrated by means of 0.1 N ferrous ammonium sulfate solution.

The end point is a very distinct change from blue to red and is sensitive to less than one-half drop of 0.1 N ferrous solution.

Stability of Reagents

Sulfuric acid - potassium dichromate:- The reagent appears to be stable indefinitely if stored under the conditions used. This fact is illustrated by the titration of measured aliquots

as shown in Table 12 and is substantiated by Williams and Reese (37).

On heating the reagent in a steam bath there is a definite and reproducible loss of oxidizing power as is illustrated in Table 13. It is also apparent in the working curves for the sterols (Figure 9) because at zero concentration of sterol the curves do not intersect the ordinate at the zero point.

Ferrous ammonium sulfate:- This reagent was indirectly stabilized by means of a lead reductor described by Duke (38). When the reductor was first prepared the ferrous ammonium sulfate solution was circulated through it three times, total volume about 15 litres, in order to ensure thorough mixing. During the first month of use there was an apparent increase in the reducing power of this solution which amounted to approximately 1%. After this period the solution remained stable throughout the course of this work.

Preparation of Working Curve

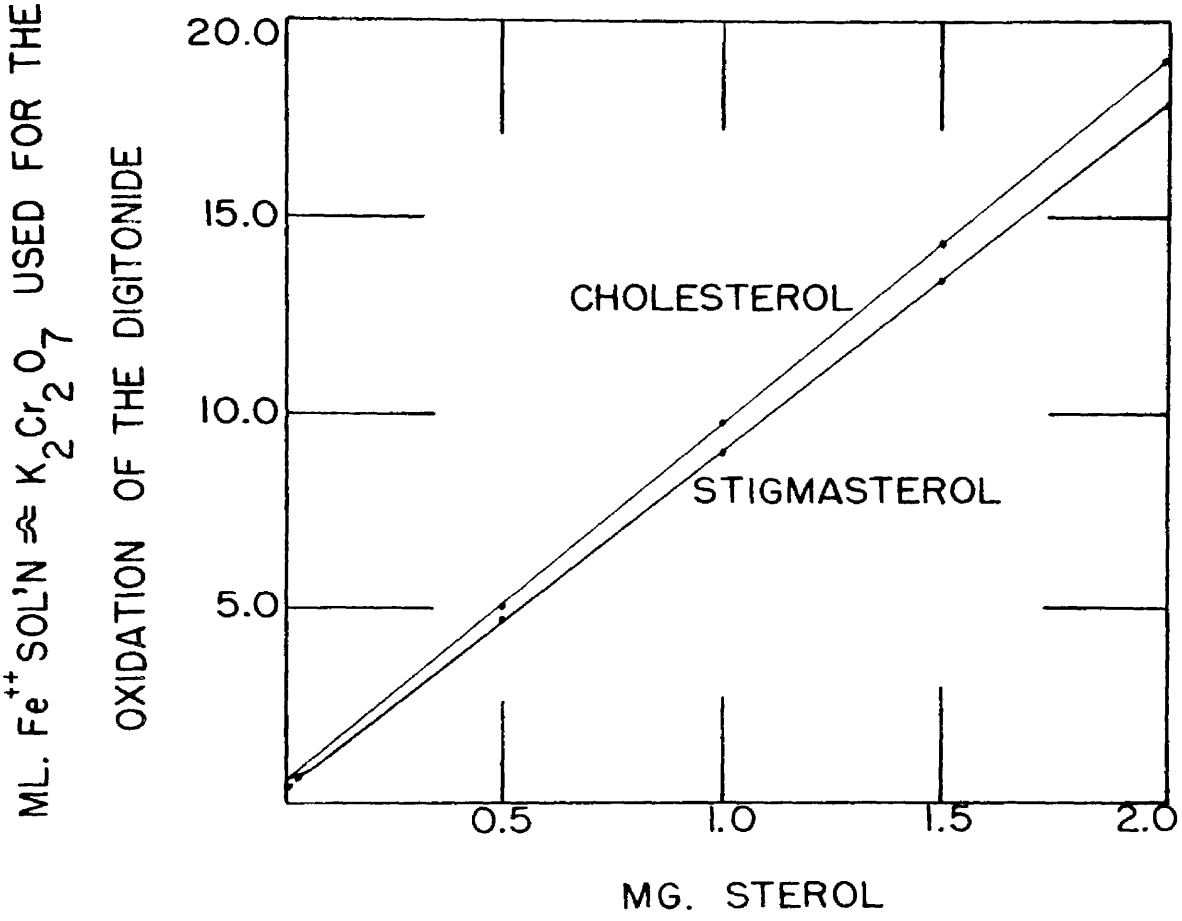
In earlier investigations it has been assumed that the oxidation proceeds with the

TABLE 13

LOSS OF OXIDIZING STRENGTH OF $K_2CR_2O_7$ - H_2SO_4

ON HEATING

Time of Heating Hours	Ml. Fe++ Sol'n.	% Recovery $Cr_2O_7^=$
0	23.10	100
1	22.97	99.43
2	22.88	99.05
3	22.73	98.33
4	22.65	98.03



WORKING CURVES FOR CHOLE-STEROL AND STIGMASTEROL

FIG. 9

exclusive formation of carbon dioxide and water and the cholesterol content has been calculated on this basis. Okey (28) assumed the formula for cholesterol digitonide to be $C_{82}H_{140}O_{29}$, M.W. 1585, and calculated that 10.62 ml. of 0.1 N potassium dichromate were equivalent to 1 mg. of cholesterol, whereas Monasterio (6), reporting no formula, calculated that 10.48 ml. of 0.1 N sodium thiosulfate were equivalent to 1 mg. of cholesterol as the digitonide.

Feeling that this assumption may not be sound, and certainly it did not hold for the conditions outlined in the present work, the author felt it better to consider the determination as being empirical and so prepared a working curve by plotting the mg. of sterol present against the ml. of ferrous solution equivalent to the dichromate used for the oxidation (Figure 9). The titration values on which Figure 9 is based are to be found in Table 14.

It is apparent, that at very low concentrations of stigmasterol the straight line relationship does not hold and determinations in

TABLE 14

OXIDATION OF KNOWN AMOUNT OF CHOLESTEROL AND STIGMASTEROL

Cholesterol

Cholesterol present mg.	Ml. Fe++ Sol'n. excess $Cr_2O_7^=$	Ml. Fe++ Sol'n. $Cr_2O_7^=$ used for oxidation
0.00	23.30	----
0.25	20.55	2.75
0.50	18.20	5.10
0.75	16.08	7.22
1.00	13.58	9.72
1.25	11.29	12.01
1.50	8.92	14.38
1.75	6.55	16.75
2.00	4.21	19.09

(Continued)

TABLE 14 (Cont'd)

OXIDATION OF KNOWN AMOUNT OF CHOLESTEROL AND STIGMASTEROL

Stigmasterol

Stigmasterol present mg.	Ml. Fe++ Sol'n. excess $Cr_2O_7^=$	Ml. Fe++ Sol'n. $Cr_2O_7^=$ used for oxidation
0.00	23.25	----
0.25	20.85	2.40
0.50	18.59	4.66
0.75	16.26	6.99
1.00	13.99	9.26
1.25	11.92	11.33
1.50	9.65	13.60
1.75	7.35	15.90
2.00	5.50	17.75

this range (0-0.1 mg. sterol) are inaccurate. This lack of linearity may be attributed to the fact that stigmasterol digitonide is more soluble in 80% ethanol than is cholesterol digitonide. If the straight line portion of the stigmasterol curve is extrapolated to zero concentration, the difference between this point of intersection and the reagent blank should give a measure of the solubility of stigmasterol digitonide. This value lies in the neighbourhood of 30 µgm. per 8.25 ml. of 80% ethanol. On the same basis, the cholesterol curve when extrapolated to zero concentration intersects the ordinate at a point which coincides with the reagent blank, therefore, cholesterol digitonide is not appreciably soluble in 80% ethanol.

In addition, the slope of the curve for stigmasterol is about 6% less than that for cholesterol. This may be accounted for by the fact that the molecular weight of stigmasterol is about 6% greater than that of cholesterol so less digitonide precipitate would be expected for equal weights of the two.

Reproducibility with other Plant Sterols

In order to check the recovery of other plant sterols as compared with stigmasterol, standard solutions of sitosterol, mixed sitosterol and stigmasterol and isolated corn sterols were prepared. Measured aliquots of these solutions were used and the recovery of the sterols, as stigmasterol, was noted (Tables 16, 17, 18).

These results would indicate that the solubility of the digitonides of these various plant sterols is practically identical and that a working curve prepared from one, i.e. stigmasterol, can be used for the determination of the others. However, discretion should be used in extrapolating to other mixtures.

Validity of Procedure

A series of samples of corn leaf tissue obtained during various stages of growth were extracted and the free sterols determined. Replications of each sample were run separately after the original extraction with hexane. From Table 19 it can be seen that the accuracy of the determination should be not less than ± 3%.

TABLE 16

RECOVERY OF SITOSTEROL FROM HEXANE SOLUTION

Tube No.	Mg. Sitosterol Present	Mg. Sitosterol Found	% Recovery
P	0.200	0.189	94.5
I	0.300	0.300	100.0
D	0.350	0.345	98.6
K	0.400	0.397	99.2
T	0.500	0.500	100.0
V	0.700	0.690	98.6
U	1.050	1.035	98.6
M	1.500	1.455	97.0

TABLE 17

RECOVERY OF MIXED STIGMASTEROL AND SITOSTEROL (50-50) FROM HEXANE SOLUTION

Tube No.	Mg. Sterols Present	Mg. Sterols Found	% Recovery
R	0.200	0.200	100.0
X	0.300	0.302	100.6
H	0.350	0.348	99.4
V	0.400	0.389	97.2
O	0.500	0.507	101.4
A	0.700	0.700	100.0
C	1.050	1.025	97.6
Y	1.500	1.478	98.5

TABLE 18

RECOVERY OF RECRYSTALLIZED CORN STEROLS FROM HEXANE SOLUTION

Tube No.	Mg. Sterols Present	Mg. Sterols Found	% Recovery
E	0.100	0.110	110.0
V	0.275	0.280	101.8
G	0.300	0.305	101.6
I	0.450	0.461	102.5
X	0.600	0.595	99.2
K	0.725	0.717	98.9
T	0.900	0.920	102.2
F	1.500	1.485	99.0

TABLE 19

PRECISION OF DATA OBTAINED FROM CORN LEAF TISSUE

Sample No.	Mg. Sterol Found	% Deviation from Mean.
19	0.665	-0.60
	0.667	-0.30
	0.677	+1.20
	0.670	+0.15
	0.669	0.00
	0.667	-0.30
13	0.440	-2.65
	0.457	+1.11
	0.454	+0.44
	0.457	+1.11
7	0.736	+0.41
	0.730	-0.41
9	0.660	0.00
	0.660	0.00
11	0.600	+0.84
	0.590	-0.84
15	0.422	0.00
	0.422	0.00
17	0.690	+1.00
	0.675	-1.00
20	0.494	+0.80
	0.485	-0.80
8	0.518	+2.20
	0.496	-2.20
10	0.383	+1.35
	0.393	-1.35

Because these replications were run side by side on the same day, a further single determination of four of the above samples was run. These four check determinations were run on the same day but about three weeks after the last of the replications in Table 19. The figures in Table 20 would indicate that the results are consistent from week to week and that there is no appreciable change in the strength of the reagents. This fact is also substantiated by the results of Table 21 which shows the recovery of known amounts of stigmasterol one month after the preparation of the working curve.

Recovery of Added Stigmasterol to Corn Extracts

In order to check the recovery of added sterol, two series of recovery checks were made. Series A in which the stigmasterol was added following the adsorption of the sample and Series B in which the stigmasterol was added before the adsorption (Table 22).

Interfering Substances

Digitonin has been reported to precipitate phosphatides from petroleum ether solution (39) and to precipitate alcohols other than the

TABLE 20

PRECISION OF REPLICATIONS RUN AFTER A 3 WEEK TIME INTERVAL

Sample No.	Mg. Sterol Found	% Deviation from Values in Table 19
7	0.770	+5.0
8	0.520	+2.6
9	0.648	-1.8
10	0.405	+4.4

TABLE 21

RECOVERY OF STIGMASTEROL FROM HEXANE SOLUTION ONE MONTH AFTER THE PREPARATION OF THE WORKING CURVE

Tube No.	Mg. Stigmasterol Present	Mg. Sterol Found	% Recovery
H	0.075	0.080	106.6
V	0.100	0.106	106.0
Y	0.300	0.306	102.0
J	0.400	0.405	101.2
W	0.600	0.607	101.2
D	1.040	1.040	100.0
R	1.095	1.095	100.0
I	1.350	1.375	101.8

TABLE 22

A

RECOVERY OF STIGMASTEROL ADDED AFTER ADSORPTION OF SAMPLE

Sample No.	Sterol Found	Sterol Added	% Recovery
9	0.660	0.250	100.0
10	0.378	0.250	98.8
12	0.520	0.250	98.0
8	0.507	0.500	95.6
6	0.503	0.500	104.2
14	0.473	0.500	101.4
11	0.595	0.750	97.7
15	0.422	0.750	99.1

(Continued)

TABLE 22 (Cont'd)

B

RECOVERY OF STIGMASTEROL ADDED BEFORE ADSORPTION OF SAMPLE

Sample No.	Sterol Found	Sterol Added	% Recovery
18	0.660	0.250	100.5
16	0.673	0.500	96.6
17	0.683	0.500	100.6
19	0.669	1.000	99.3
20	0.490	1.000	98.4

sterols (40, 41).

It was found that under the conditions used in this work for the digitonin precipitation no precipitate would form with lecithin until more than 5 mg. were present. The addition of 1 mg. and 5 mg. of lecithin to samples of corn leaf tissue, both before and after adsorption, resulted in no increase in the amount of digitonide precipitate.

Using oleyl alcohol and stearyl alcohol as representative members of the higher alcohols it was found that no precipitation occurred under the above conditions until more than 16 mg. of these alcohols were present. The results obtained with added lecithin and the alcohols are found in Table 24.

In view of these results and the size of the aliquots used in the determination itself it would appear that interference from these sources is unlikely in normal plant tissue.

TABLE 24

THE EFFECT OF ADDED IMPURITIES ON THE RECOVERY OF STEROLS

Mg. Sterol in Sample	Added Impurity	Mg. Sterol Found
None	5 mg. Lecithin	None
None	10 mg. Lecithin	0.053
0.665	1 mg. Lecithin	0.669
0.667	5 mg. Lecithin	0.667
None	4 mg. Alcohols *	None
None	16 mg. Alcohols *	None
0.485	4 mg. Alcohols *	0.483
0.485	16 mg. Alcohols *	0.488

* 50-50 mixture of stearyl and oleyl alcohols

IV APPLICATION OF THE METHOD

During the summer of 1950 Golden Bantam sweet corn was grown in order to obtain tissue samples of the various parts of the plant throughout the growing season. The plots were located at Hespeler, Ontario, Canada, in a light sandy loam soil which was seeded with winter rye in the fall of 1948 and which was allowed to mature in 1949, was then cut and ploughed under while in the swath. Seeding was done on June 3rd, 1950. At approximately eight day intervals samples were harvested until the set of seed. As the plants developed they were divided into various fractions, i.e. tassell, silk, stalk, cob husks, etc. At one stage of development, July 26 and 27, samples were obtained at four hour intervals in order to study any diurnal variations.

The percent moisture recorded was obtained by weighing the fresh sample when harvested and subsequently weighing the air dried material. The lipid and sterol contents were calculated on this air dry weight and not on a moisture free basis. The percent lipid was determined by the

difference in weight of the air dry sample before and after exhaustive hexane extraction in a soxhlet type extractor. After extraction the solvent was removed from the samples by drying in an oven at 63-65°C. for 48 hours and then allowing them to come to equilibrium with the air (usually required 4 - 7 days). The sterol analyses were carried out on the hexane extracts by the oxidative method outlined and the results are tabulated in Tables 25 and 26.

A number of interesting observations can be made from Table 25. In the first place, the growth curve of the corn leaves follows the well known form as is seen in Figure 10. Secondly, the total lipids reach a peak rather early in the development of the corn and then fall very markedly. This fact is illustrated in Figure 11. As this decrease in total lipids coincides with the very rapid growth of the young cobs it might be postulated that they are reentering the metabolic cycle in some form, perhaps converted to carbohydrate, and are then utilized for the development of these cobs. Thirdly, the sterols are retained in the leaf for a much longer

TABLE 25

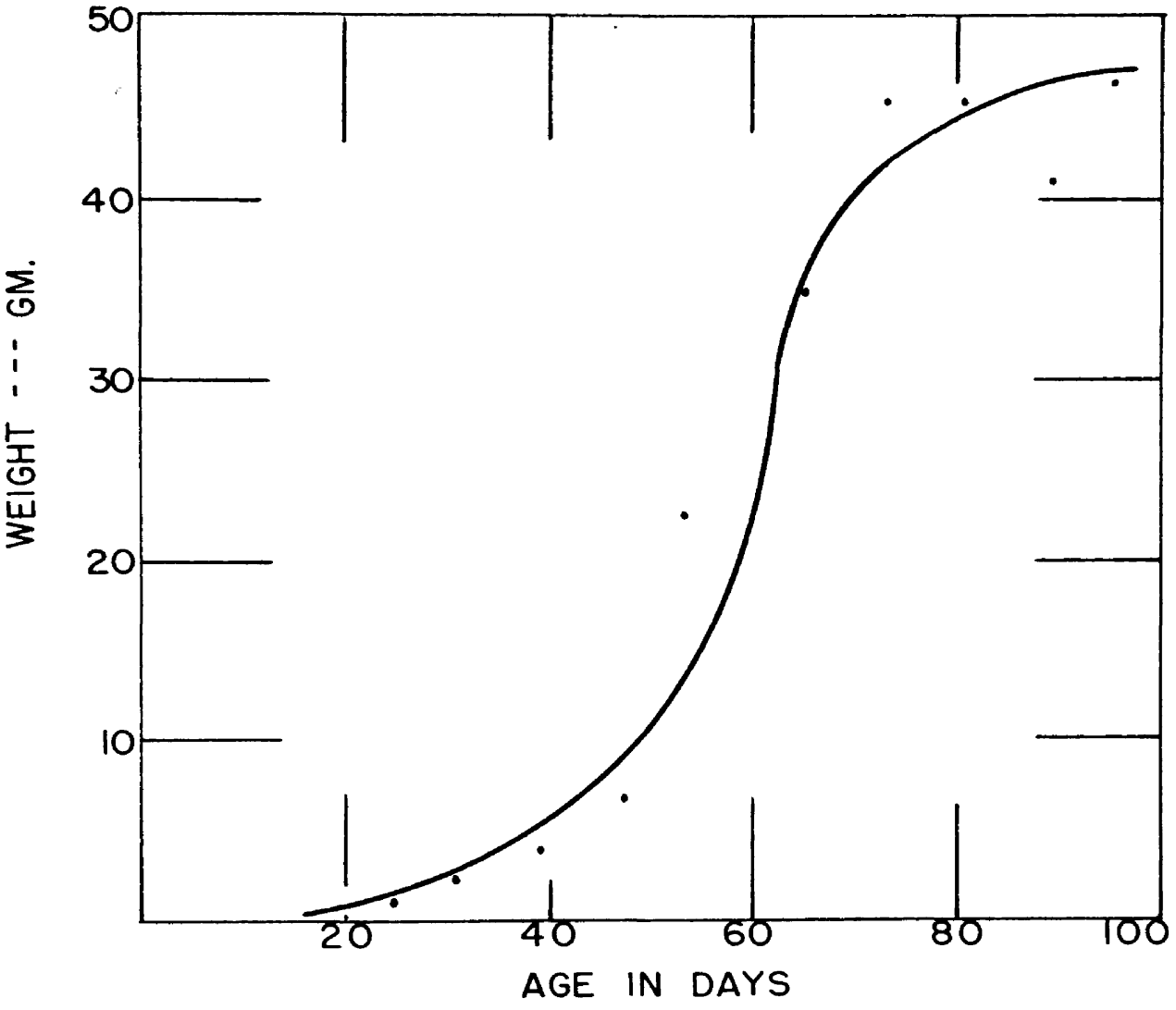
CHANGES IN COMPOSITION, WITH PARTICULAR REFERENCE TO THE STEROLS, OF CORN LEAF TISSUE THROUGHOUT THE GROWING SEASON

Age of Plant Days	Fresh Weight Gms.	Air Dry Weight Gms.	No. of Plants	% Moisture	% Lipid	Total Dry Weight per Plant Gms.	Mgm. Sterol per gm. Tissue	Mgm. Sterol per Plant
9	107.0	11.0	344	90.0	5.4	0.03	1.250	0.04
17	285.5	32.0	216	89.0	6.1	0.15	0.880	0.13
24	678.0	78.0	104	89.0	5.6	0.75	1.006	0.75
32	504.0	54.0	35	89.0	5.2	1.50	0.915	1.41
39	722.0	78.0	20	90.0	7.4	3.9	0.633	2.47
47	990.0	124.0	18	89.0	6.4	6.9	0.825	5.69
53	669.0	113.0	5	83.2	6.8	22.6	0.520	11.8
65	502.0	106.0	3	79.0	5.2	35.3	0.673	23.8
73	649.0	139.5	3	78.5	1.4	46.5	0.682	32.0
81	407.0	93.5	2	77.0	0.7	46.7	0.660	30.0
89	327.0	82.5	2	74.5	1.32	41.1	0.669	27.5
96	485.0	143.5	3	70.0	0.4	47.7	0.490	23.3

TABLE 26

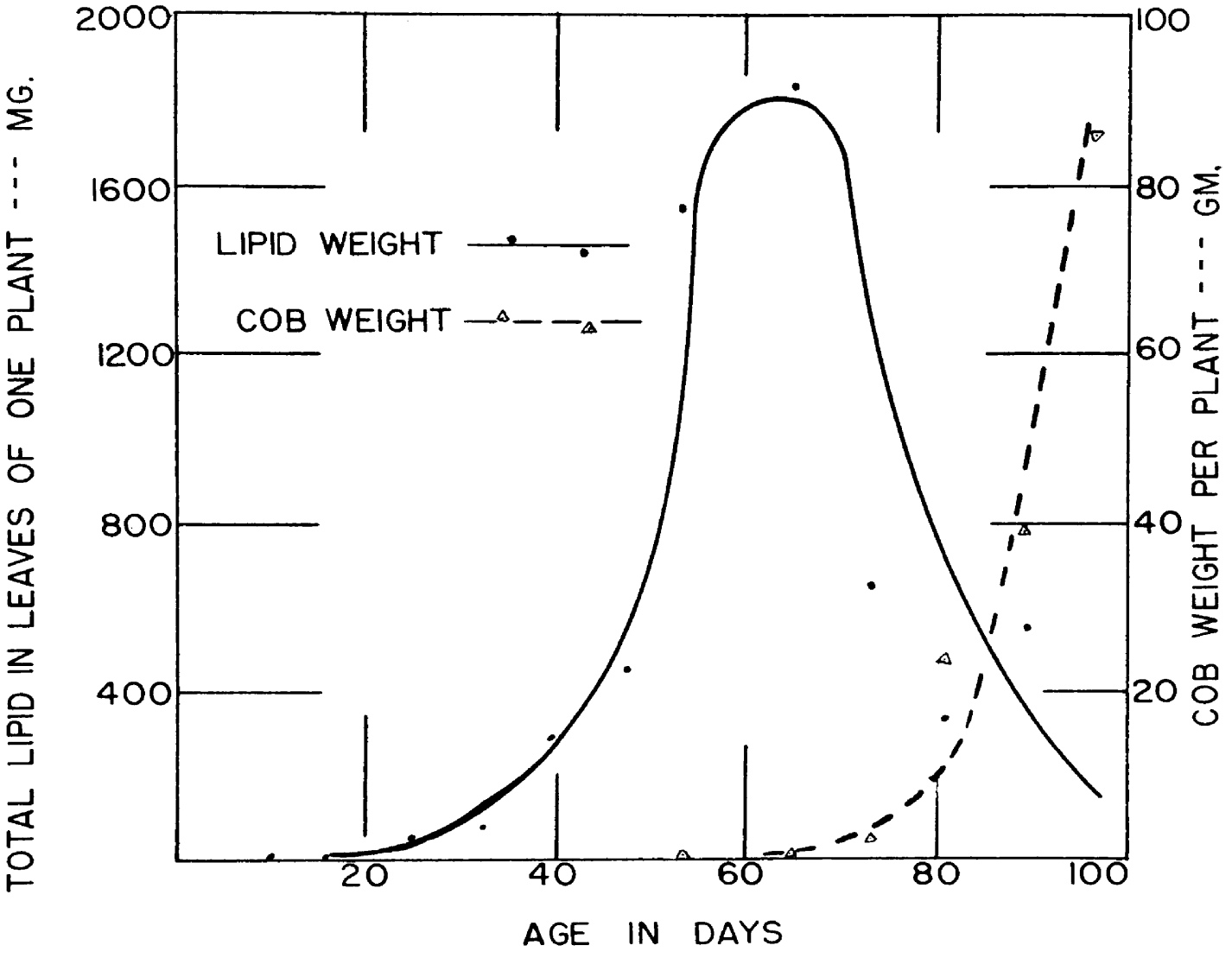
CHANGES IN COMPOSITION, WITH PARTICULAR REFERENCE TO THE STEROLS, OF CORN LEAF TISSUE OVER A 24 HOUR PERIOD

Time of day of Sampling	Fresh Weight Gms.	Air Dry Weight Gms.	No. of Plants	% Moisture	% Lipid	Total Dry Weight per Plant Gms.	Mgm. Sterol per gm. Tissue	Mgm. Sterol per Plant
Midnight	769.0	112.0	5	86.0	6.1	22.5	0.473	10.6
4:00 a.m.	804.0	106.0	5	87.3	6.3	21.2	0.595	12.6
8:00 a.m.	669.0	113.0	5	83.2	6.8	22.6	0.520	11.8
Noon	846.0	141.0	5	83.5	7.1	28.2	0.452	12.7
4:00 p.m.	645.0	113.0	5	82.5	7.1	22.6	0.473	10.7
8:00 p.m.	754.0	118.0	5	84.4	7.1	23.6	0.422	10.0



GROWTH CURVE OF CORN LEAVES

FIG. 10

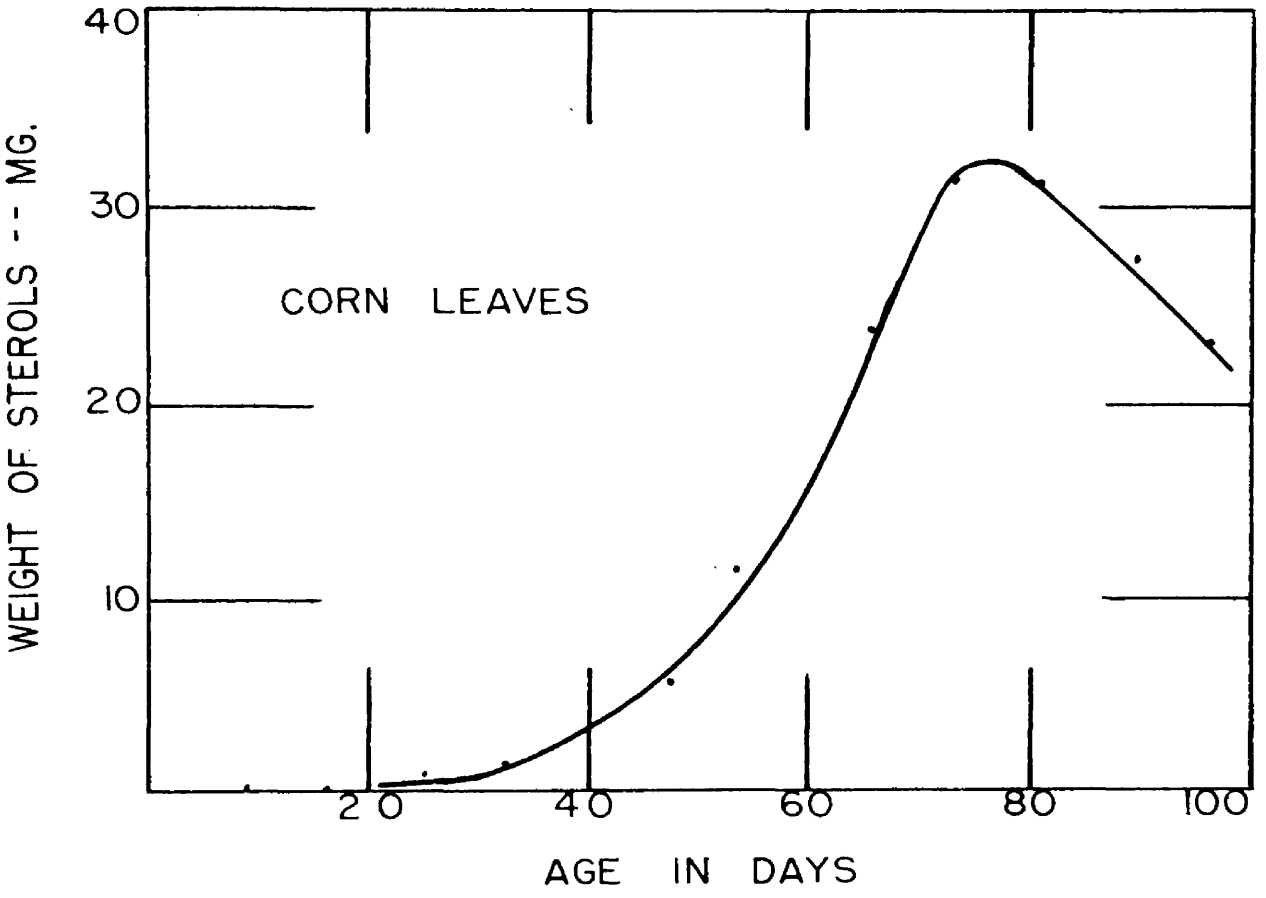


COMPARISON OF TOTAL LIPID OF LEAVES WITH AGE AND COB WEIGHT

FIG. 11

period but finally they too begin to disappear as is seen in Figure 12. Fourthly, there can be little doubt that the corn leaf has the ability to synthesize sterols up to about the 75th day of growth but that following this period the synthesis is definitely limited and does not parallel their destruction. Fifthly, the rate of synthesis of the sterols may not quite keep pace with the rate of growth of the corn leaf because there seems to be a slight decrease in the percentage value.

From Table 26 it would appear that there is little variation in the sterol levels in corn leaves over a 24 hour period.



DEVELOPMENT OF FREE STEROLS WITH AGE

FIG. 12

V CONCLUSIONS

1. The quantitative estimation of mixed sterols through reactions involving the hydroxyl group does not seem feasible on a micro or semi-micro basis.
2. The quantitative estimation of mixed sterols by nephelometry does not appear feasible under the conditions studied unless an intimate knowledge of the individual sterols present is available.
3. Nephelometry might easily be adapted for a rapid micro estimation of individual sterols.
4. Estimation of the reducing sugars liberated by the hydrolysis of the digitonides yields results of the desired sensitivity but lacks the required precision. The lack of precision appears to be due to poor control of the boiling rate during hydrolysis.
5. The oxidation of the digitonide by dichromate followed by the quantitative estimation of the excess dichromate yields consistent results of good precision and sensitivity.
6. The use of Nicloux's reagent for the oxidation is not necessary.

7. Under the conditions used the oxidation does not proceed to the exclusive formation of carbon dioxide and water and cannot be considered a stoichometric reaction, thus the determination is considered to be purely empirical.
8. The precision of the method is better than ± 3% and samples containing as little as 0.15 mg. of sterol can be used.
9. The total lipid fraction of the corn leaves reached a maximum in about 65 days and then decreased very markedly. This decrease just precedes the very rapid development of the cobs.
10. The sterol fraction of the corn leaves reached a maximum in about 75 days and their subsequent decrease was not as marked as that of the total lipid fraction.
11. These results are based on only one year's crop and should be further substantiated.

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