BLOOD SERUM LIPIDS OF THE CHICK AS AFFECTED BY SAFFLOWER OIL: NATURAL AND PARTIALLY HYDROGENATED

by

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CHAPTER I

INTRODUCTION

Investigations in humans and animals have established evidence that the amount and kinds of fat in the diet influence the level of various serum lipids.

Certain oils, those which are highly unsaturated, produce lower serum cholesterol levels than the more saturated oils or hydrogenated fats in both humans (Kinsell et al., 1953; Bronte-Stewart et al., 1956; Beveridge et al., 1956; Malmros and Wigand, 1957) and in animals (Okey and Lyman, 1957; Lambert et al., 1958; Hegsted et al., 1960).

Numerous workers have suggested that various factors in fats may be responsible for lowering serum cholesterol. Ahrens et al. (1957) fed formula diets containing natural and hydrogenated vegetable oils (corn, safflower, and cottonseed) to patients and reported a linear correlation between the degree of saturation (as measured by the iodine value) of a fat with the level of serum cholesterol. Jones et al. (1956) observed the same results with chicks fed corn and cottonseed oil.

The ratio of polyunsaturated to saturated fatty acids was reported by Keys et al. (1959) as the factor in dietary fats responsible for raising or lowering serum cholesterol in humans. Two to three grams of an oil containing a high content of linoleic acid was required to offset the cholesterol-raising effect of one gram of a highly saturated fat. Monounsaturated fatty acids had little effect in either

raising or lowering the level. Hegsted et al. (1960) observed similar results when mixtures of fats containing varied proportions of saturated, monounsaturated, and polyunsaturated fatty acids were fed to chicks.

More recent investigations in humans by McOsker et al. (1962) indicated that partially hydrogenated vegetable fats with P/S ratios greater than 0.5 produced serum cholesterol levels equal to that obtained when highly unsaturated cottonseed oil was fed.

Beveridge et al. (1959), Ahrens (1957), and Horlick and Craig (1957) showed that fats containing various proportions of saturated fatty acids of different chain lengths varied in their hypercholesteremic effect in man. Those fats containing saturated fatty acids in the range C_6 to C_{14} appeared to raise serum cholesterol levels more than fats with saturated fatty acids in the range C_{16} to C_{20}. Grande (1962) fed three fat mixtures composed of saturated fatty acids of different chain lengths to dogs at 40% of the dietary calories. Those mixtures containing fatty acids with a chain length of 12 to 14 carbon atoms raised serum cholesterol to a greater extent than fat mixtures containing 8 to 10 or 16 to 18 carbon atoms. Vegetable oils used in the test were coconut, olive, and partially hydrogenated corn oil.

Other investigators have favored a concept of essential fatty acid deficiency. When Kinsell et al. (1958) fed purified ethyl and glycerol esters of linoleic acid to patients, blood lipid levels were lowered; whereas, esters of oleic acid did not have the same effect. Sinclair (1956) proposed that when sufficient amounts of the essential fatty acids are not present in the diet, cholesterol forms abnormal

esters which may be less readily disposed of, resulting in raised blood cholesterol levels.

Also, under discussion at present is the possible effect on cholesterol metabolism of fatty acid isomers not found in natural fats and oils but which appear as a result of hydrogenation. Melnick et al. (1954) reported changes which occur during the hydrogenation of oil. He stated that concentration, per se, of saturated fatty acids increases very little, but that preferential hydrogenation to a monoene trans-isomer of the same chain length is the major modification of the linoleic acid content. This trans-oleic acid, known as elaidic, contributes to the firm consistency of the hydrogenated oil due to the acid's higher melting point in contrast to that of the natural cis-acid.

Privett et al. (1955), Holman and Aaes-Jorgensen (1956), and Mattson (1960) observed that trans-acids did not replace cis-acids in remedying essential fatty acid deficiencies in animals. Holman (1951) suggested that the isomers of the essential fatty acids may act as metabolic antagonists to the natural form. Mattson, however, demonstrated that fats containing the trans-isomers of linoleic, including elaidic acid, did not interfere with the essential fatty acid activity of the all cis-form. The possible effects of the trans-fatty acids on other metabolites remain unclear.

Kahn (1957), Alfin-Slater et al. (1957), Johnston et al. (1958), and Allen et al. (1960) demonstrated that the animal organism is capable of efficiently metabolizing trans-isomers which appear to be equivalent to their precursors in promoting growth, maturation, and reproduction.

These studies, although not rejecting the hypothesis of Sinclair referred to above, do indicate that if trans-fatty acids contribute to increased serum cholesterol, it is not due to the inability of the animal to metabolize them. McOsker's et al. (1962) investigation of patients fed partially hydrogenated fat containing a high content of total trans-fatty acids (21%) but with a P/S ratio above 0.5, did not show an elevation in serum cholesterol levels. The authors stated that consumption of hydrogenated fat containing a higher percentage of trans-acid than was used in their study might result in increased serum cholesterol concentrations.

Gofman et al. (1950) in studies with both humans and rabbits, and Orma (1957) and Nishida et al. (1958) with chicks, reported that an increase in the beta-lipoprotein fractions (low density fractions due to increased lipid content) of the serum accompanied hypercholesterolemia. When Nichols et al. (1957) fed diets containing large amounts of animal fat to humans, they observed a pronounced elevation of cholesterol-rich S_f 0 to 12 and S_f 12 to 20 beta-lipoprotein fractions over those obtained on a low fat diet or one containing large amounts of vegetable oil. S_f 20 to 400 beta-lipoprotein fractions (those having increasingly low specific gravity) were raised with a low fat, high carbohydrate diet. The effect of dietary safflower oil, linoleic acid rich, and of linoleic acid, per se, on the ratio of beta-lipoproteins to alpha-lipoproteins (high density) was studied by Labecki et al. (1958) in human serum. Following the feeding of either the oil or acid, they observed a lowering of total serum cholesterol and an accompaning lower beta to alpha ratio.

Portman et al. (1956) found that serum cholesterol and S_f 12 to 100 beta-lipoprotein concentrations were increased in monkeys when hydrogenated cottonseed oil was substituted for corn oil in the diet but only when dietary cholesterol also was fed. Similar studies were reported by Leveille et al. (1960) with chicks. When the diet contained cholesterol, the more saturated coconut oil produced higher plasma cholesterol levels than corn oil. Serum protein fractions were unaltered by a change in dietary fats, but addition of cholesterol to the diets resulted in an increase in the $alpha_2$ and $alpha_3$ globulin serum fractions. Birds receiving added cholesterol had significantly more lipids bound to beta-lipoprotein than animals not so fed. These investigators reported, however, that neither the amount of lipid bound to beta-lipoprotein nor the beta to alpha ratio was affected significantly by a difference in amount and kinds of fat fed.

The mechanisms by which the highly polyunsaturated fats lower the lipid levels of the blood are poorly understood. Various investigators have reported on a relationship of the polyunsaturated fatty acid components to the transport and metabolism of blood cholesterol. Mukherjee et al. (1958), Sinclair (1956), Kelsey (1941), and Aftergood et al. (1957) proposed that, normally, cholesterol esterified preferentially with unsaturated fatty acids for transport to the liver. Gordon et al. (1957), Hellman et al. (1957), Haust and Beveridge (1958), and Lewis et al. (1961) have shown that administration of fats rich in polyunsaturated fatty acids cause an increased excretion of neutral sterols and bile acids in the feces.

They report this could be the result of the degradation of cholesterol to the bile acids at an accelerated rate in the liver, or the result of decreased reabsorption of these acids from the gastrointestinal tract, or both mechanisms could be involved. Studies by Goldsmith et al. (1960) indicated that polyunsaturated fats cause a change in the intestinal flora, which modifies the structure of the bile acids and neutral sterols. This change in molecular structure is presumed to affect reabsorption leading to lowered body cholesterol.

Safflower seed oil, which is a rich source of the essential fatty acid linoleic with a high polyunsaturated to saturated fatty acid ratio, lends itself well to a study of the effects of these factors on the lipid levels of the blood.

In the process of hydrogenation of safflower oil,[1] as used in the present study, the polyunsaturated fatty acids, linoleic (C_{18}) and linolenic (C_{18}), decreased from 74 to 4% with the synthesis of 62% of the trans-isomer, elaidic (C_{18}), and an increase of only 12% in saturated acids of 16 to 18 carbons. Oleic (C_{18}) acid remained unchanged at 13%.

The chicken, which is omnivorous, was selected as the experimental animal for this study because, like man, it has a natural mechanism for deposition of cholesterol with age. Demonstration of increasing concentrations of cholesterol and other lipids in arterial walls of man, associated with his longer life span and presumably less desirable national dietary, is at present cause for widespread concern.

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[1]Natural oil and hydrogenated product were kindly supplied by the Pacific Vegetable Oil Corporation, San Francisco, California.

This study was undertaken to determine whether the reduction of serum cholesterol, which is normally very high in the chick the first few weeks after hatching (Entenman et al., 1940, and Rodbard et al., 1951), is associated with changes in serum protein and lipoprotein fractions and their relative concentrations as the amount and kinds of dietary fat are changed. The diets contained no cholesterol which by itself has been shown to affect the lipid levels of the chick (Katz and Stamler, 1953). Lambert et al. (1958), Avigan and Steinberg (1958), and March and Biely (1959) observed in studies on the rabbit, rat, and chick, respectively, that the effect of dietary fats on circulating cholesterol could be more clearly differentiated when dietary cholesterol was not fed.

CHAPTER II

EXPERIMENTAL PROCEDURE

Animals and Their Rearing

Three lots of White Leghorn cockerels of the Kimber strain (22 per lot, 2 replicates), produced by the University of Arizona Poultry Research Center, were subjected to dietary treatment over an eight-week experimental period. Replicates were allocated to separate pens of electrically heated, wire-floor batteries in such a manner as to equalize environmental effects on each treatment. All chicks were identified by numbered wingbands and placed on experiment at one day of age.

The chicks were weighed by replicate in the beginning and individually each week thereafter. Feed and water were supplied *ad libitum*, and feed consumed per replicate was recorded weekly.

Dietary Treatments

Dietary treatments were: (1) a "fat-free" diet with no added cholesterol, (2) diet 1 with 10% by weight of partially hydrogenated safflower oil substituted for an equal weight of cerelose, (3) diet 1 with 10% by weight of safflower oil substituted for an equal weight of cerelose. Percentage composition of the diets is shown in Table 1. Diet 1, a modification of the isolated soybean protein diet of Couch *et al.* (1957),

contained 27% protein, 0.45% fat and 58% carbohydrate (cerelose) with 1023 calories of productive energy per pound of feed (calculated on the basis of values of Titus, 1955). Treatments 2 and 3 contained 27% protein and 1179 calories of productive energy per pound. All diets were stored at refrigeration temperature (40 F.) after mixing, and fresh feed was placed in troughs daily to minimize oxidation.

The fatty acid composition and iodine numbers of the natural and partially hydrogenated safflower oil are given in Table 2.

Blood Samples

At the beginning of the experiment, blood was drawn by cardiac puncture from 10 additional day-old chicks which were sacrificed and the blood pooled for initial analysis. Thereafter, a postabsorption (12-hour food withdrawal) blood sample, obtained by venipuncture of the wing with a scalpel blade, was collected weekly in capillary tubes from each chick for separate analysis. After two to three hours at room temperature, the blood was centrifuged for 10 minutes at 2500 rev/min. Aliquots of 0.04 ml of the resultant clear serum were transferred to 3.0 ml tubes (previously calibrated at 1.0 ml) and frozen at -12 F. for analysis later.

Analysis of Serum Cholesterol

Samples of sera were analyzed in triplicate for total cholesterol by Adamson's (1960) micromodification of the method of Pearson et al. (1953). This modified method reduced the serum required to 0.04 ml and added a preliminary deproteinization and extraction with a 1:1

Table 1

PERCENTAGE COMPOSITION OF DIETS

Ingredient	Treatment 1	2	3
ADM Assay Protein[1]	32.0	32.0	32.0
Cerelose[2]	52.2	42.2	42.2
Vitamin E Solution[3] (in alcohol)	0.3	0.3	0.3
Mineral Mix[3]	8.0	8.0	8.0
Solka Floc (Cellulose)	3.0	3.0	3.0
Vitamin Mix[3]	4.0	4.0	4.0
Choline Chloride Solution	0.5	0.5	0.5
Partially Hydrogenated Safflower Oil	---	10.0	---
Liquid Safflower Oil	---	---	10.0

[1]Archer Daniels Midland Company, Cincinnati, Ohio

[2]Powdered 2001, Corn Products Company, New York, New York

[3]Dannenburg, W. N., B. L. Reid, and J. R. Couch,1955. An inorganic chick growth response. Poultry Sci. 34:1023.

Table 2

FATTY ACID COMPOSITION,[1] IODINE VALUES,[2] AND P/S RATIOS[3] OF NATURAL AND PARTIALLY HYDROGENATED SAFFLOWER OIL

	Natural	Partially Hydrogenated
Fatty Acids		
Caprylic	----	trace
Capric	----	trace
Lauric	trace	0.5%
Myristic	0.1%	0.3%
Palmitic	5.8%	7.2%
Stearic	2.5%	13.4%
Palmitoleic	trace	----
Oleic	13.7%	12.7%
Elaidic	----	62.0%
Linoleic-Linolenic	77.8%	3.9%
Iodine Value	142.2	74.3
P/S Ratio	9.26	0.18

[1]Percentage by weight as determined by the Pacific Vegetable Oil Corporation, San Francisco, California.

[2]Determined by the Pacific Vegetable Oil Corporation, San Francisco, California.

[3]Percentage polyunsaturated fatty acids divided by percentage saturated fatty acids.

redistilled acetone-ethanol mixture before color was developed. Samples of sera were removed from the freezer just prior to cholesterol determination and brought to 27 - 30 C. temperature. Four-tenths milliliter of acetone-ethanol was added to each of three tubes of sera at a time. The tubes were buzzed to separate the protein precipitate into fine particles, dipped into a beaker of boiling water for approximately five seconds to bring the contents to a boil, then placed immediately into a beaker of cool water. The contents were brought to 1.0 ml volume with acetone-ethanol, capped, buzzed, and centrifuged at 2500 rev/min for 20 minutes to pack the precipitate tightly in the bottom of the tube. A 0.2 ml aliquot of supernatant was then transferred into 1.0 ml tubes and placed in a 55 C. vacuum oven for one hour for evaporation of the solvent. Ten samples were read in triplicate at one time. When samples were dried in larger numbers, the remaining tubes were capped and stored for reading later.

Development of color. A stock cholesterol standard and a solution of p-toluenesulfonic acid in glacial acetic acid were prepared in advance and stored at refrigerator temperature. The stock cholesterol standard was prepared by dissolving 200 mg cholesterol[1] in approximately 50 ml reagent-grade glacial acetic acid. The mixture was warmed to dissolve the cholesterol, transferred quantitatively into a 100 ml volumetric flask, and brought to volume at room temperature with glacial acetic acid. The p-toluenesulfonic acid solution was prepared by

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[1] M.P. 148 - 150 C. Matheson Company, Inc., East Rutherford, New Jersey.

dissolving 12 g of reagent-grade p-toluenesulfonic acid in reagent-grade glacial acetic and bringing to 100 ml volume at room temperature. The stock cholesterol standard and the p-toluenesulfonic acid solution were brought to 27 - 30 C. before using.

Two blanks and three standards were prepared for each set of serum samples to be read. Standards were prepared by pipetting 10 µl of redistilled water into each 1.0 ml tube. Ten µl of the stock cholesterol standard were then added. Twenty µl of 50% acetic acid were transferred to each blank and serum tube. To all tubes were added, in turn, 50 µl p-toluenesulfonic acid solution, 150 µl acetic anhydride, and 20 µl concentrated sulfuric acid. They were then buzzed, capped, and allowed to stand at room temperature for color development.

Reading of samples. Samples were read between 30 to 90 minutes after addition of the sulfuric acid, the period of time of maximum color intensity. Samples were transferred to microcuvettes and read at 550 mµ in a Beckman DU spectrophotometer. Standards were read before the unknowns.

Three cholesterol values were obtained for each sample of serum by using the following methods of calculation: (1) average transmittance readings for each of the three tubes were averaged and converted to cholesterol, (2) average of all readings, without regard to tube, converted to cholesterol, and (3) each reading converted to cholesterol, without regard to tube, and averaged. Values obtained by these methods were shown to be in close agreement. The second method was found not only to provide as accurate a method of calculation as the others, but

was the most convenient to use.

Conversion of transmittance readings. Total cholesterol values in mg per 100 ml serum were derived as shown below:

$$\text{cholesterol, mg/100 ml} = \frac{\text{transmittance reading of sample}}{\text{transmittance reading of standard}} \times 250$$

Electrophoretic Fractionation of Serum Proteins and Lipoproteins

Serum obtained from one additional chick per treatment at five, six, seven, and eight weeks of age, was used for separation of serum protein and lipoprotein fractions by electrophoresis. A freeze-dried, pooled human serum[1] provided a constant electrophoretic pattern with each analysis.

Paper electrophoresis was carried out with a Beckman-Spinco-Durrum-type cell and a barbiturate buffer (pH 8.6, ionic strength 0.075).

Separation of serum protein. Six µl of serum were applied to each filter paper strip, and after separation, the fractions were stained with methanolic bromophenol blue according to the Spinco Electrophoresis Serum Protein Analysis, Procedure B.[2] This method was lengthened with respect to time of operation of the cell, from 16 to 17

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[1]The Warner-Chilcott Blood Chemistry Manual; Part I. Versatol and Typical Procedures in Electrolyte Chemistry, Warner Chilcott Laboratories, Morris Plaines, New Jersey.

[2]Spinco Model R Paper Electrophoresis System Instruction Manual, Spinco Division, Beckman Instruments, Inc., Palo Alto, California.

hours. Electrophoretic patterns showed six peaks as in similar patterns obtained by Bieri and Pollard (1959) and Vanstone et al. (1955). All fractions following the albumin peak were designated as globulins. Since the chick globulin fractions were not clearly defined, as was also observed by Bieri and Pollard, A/G ratios only, were calculated for interpretation of the electrophoretic patterns.

Separation of lipoproteins. Twenty µl of serum were applied to each paper strip, and after separation the fractions were stained with Ciba fat red 7B dye as described by Straus and Wurm (1958). The relative amount of each fraction was determined by photometric scanning of the stained strips. Detailed procedures for separation of serum proteins and lipoproteins are given in the Appendix (page 40).

Statistical Treatment

The data were treated by an analysis of variance (Duncan, 1955). Analysis of variance was made for a two way classification with unequal subclass numbers. The mean square for among chicks in pens was divided by the harmonic mean of the number of chicks per group, and the analysis of the diets was made on the basis of mean values per group (Snedecor, 1956). While this method is generally used for fixed effects only, it was used as the basis of analysis in the present study rather than the least squares method because of simplicity of calculation.

In a replicated design of this type, the Replicate X Diet interaction is usually the estimate of error variance for testing diet

effect, but because of the small number of degrees of freedom in this estimate, it was decided to use the sampling error. In most cases, it was observed that the experimental error was not significantly larger than the sampling error and, in some cases, smaller.

Analysis of variance showed that replicate differences in serum cholesterol values and weight gains were small. It was concluded, therefore, that in future study, chicks receiving the same treatment do not need to be arranged in replicates at different levels in the brooder but can be caged together.

Statistical differences were indicated by number superscripts. Any means followed by the same or no superscript were not significantly different.

CHAPTER III

RESULTS AND DISCUSSION

Day-old chicks, weighed by replicate at the beginning of the experiment, averaged 38 g per chick. After the first week, chicks were weighed individually and mean values of weekly weight gains for each dietary treatment are shown in Table 3.

Chicks in treatment 3, fed 10% safflower oil, showed the greatest weight gains during each of the seven-week-periods. The chicks fed the "fat free" rations, in treatment 1, gained the least.

Analysis of variance (Table 3a) revealed significant differences in weekly weight gains due to dietary treatment. Weight gains of chicks in treatment 3 from four to seven weeks of age were shown to be significantly different from those in treatments 1 and 2. No significant differences in weight gains were shown between treatments 1 and 2 during these same periods.

The relationship of weight gain to the level and types of fat in the three dietary treatments is shown in Table 4. Increasing the energy level of the diets in treatments 2 and 3 over treatment 1 by substituting 10% fat for an equal weight of cerelose resulted in lower feed consumption (determined primarily by the energy level of the ration [Hill and Dansky, 1954]) and an increase in feed efficiency (feed required per unit of weight gain). Feed consumption of chicks on

Table 3

MEAN GRAM GAIN IN WEIGHT OF CHICKS TWO TO EIGHT WEEKS OF AGE*

Treatment	Weeks on Diet						
	2	3	4	5	6	7	8
1	59.2[1]	76.9[1]	92.4[1]	108.3[1]	123.4[1]	123.8[1]	121.5
2	68.0	93.5[2]	89.4[1]	113.8[1]	125.6[1]	112.1[1]	127.5
3	76.2[2]	97.4	109.3[2]	131.0[2]	150.0[2]	139.5[2]	138.4
±SE	3.51	3.33	3.51	3.41	3.85	4.61	(ns)

*Significant differencesare indicated by number superscripts. Any means followed by same or no superscript are not significantly different ($P < 0.05$).

Table 3a

ANALYSIS OF VARIANCE (MEAN SQUARES)

Source of Variation	df	Weeks on Diet 2	3	4	5	6	7	8
Replicates	1	12.4	75.5	234.1	0.1	37.1	62.2	2244.6
Diets	2	143.6*	236.9*	229.1*	280.6*	434.1*	378.3*	146.9
Replicates X Diets	2	31.6	10.0	72.9	36.7	69.5	17.2	111.1
Among Chicks	‡	27.5	23.5	26.4	24.4	31.3	45.2	69.3

*Significant difference ($P < 0.05$).

‡Due to losses in blood samples number varied slightly with df within pens ranging from 51 to 59.

Table 4

EFFECT OF DIET ON WEIGHT GAIN, FEED CONSUMPTION, FEED EFFICIENCY, AND CALORIES REQUIRED PER GRAM GAIN IN WEIGHT OF CHICKS TWO TO EIGHT WEEKS OF AGE

Age in Weeks	Treatment											
	1	2	3	1	2	3	1	2	3	1	2	3
	Weight Gain g			Feed Consumption g			Feed Efficiency g			Cal/g Gain in Weight		
2	59.2	68.0	76.2	102.4	107.5	105.0	1.73	1.58	1.38	3.89	3.95	3.45
3	76.9	93.5	97.4	164.0	155.5	160.9	2.13	1.66	1.65	4.79	4.15	4.13
4	92.4	89.4	109.3	219.5	194.8	197.3	2.38	2.18	1.81	5.35	5.45	4.52
5	108.3	113.8	131.0	224.3	189.1	196.6	2.07	1.66	1.50	4.66	4.15	3.75
6	123.4	125.6	150.0	306.2	261.6	294.3	2.48	2.08	1.96	5.58	5.20	4.90
7	123.8	112.1	139.5	320.7	280.0	325.5	2.59	2.50	2.33	5.83	6.25	5.82
8	121.5	127.5	138.4	390.2	333.0	371.6	3.21	2.61	2.68	7.22	6.52	6.70

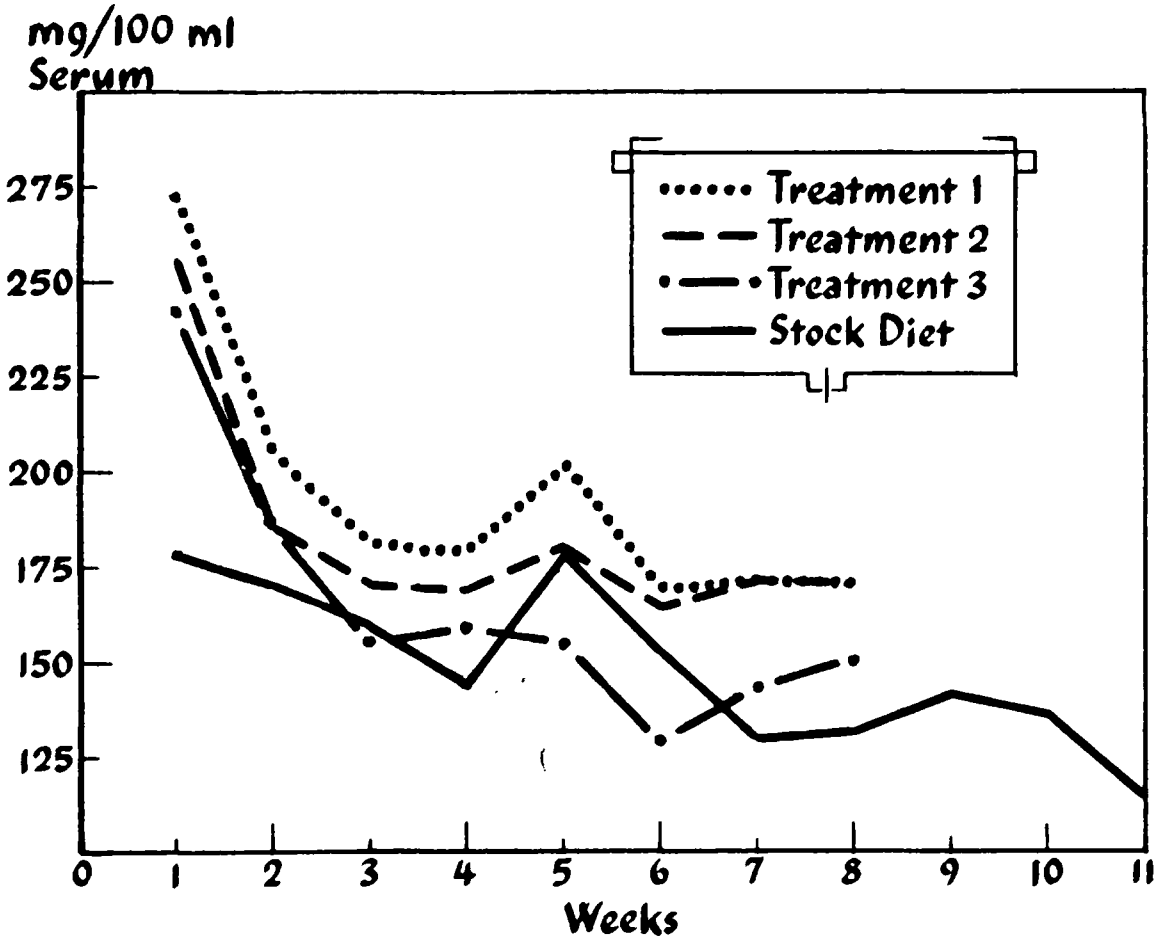
treatment 2 was lower than that of chicks on treatment 3 for which no explanation could be found. Energy levels of the two diets were equal, as calculated, and feed efficiencies did not differ significantly. A high protein level (27%) was maintained in the diets to insure similar absorption of the two fats. Weight gain studies by Dam et al. (1959) and March and Biely (1959) showed that chicks absorbed hydrogenated fats as readily as the highly unsaturated oils when diets contained 26% protein. The caloric intake required to produce one gram gain in weight (determined by multiplying feed efficiency by productive energy) was not significantly different among the three treatments. This indicated that the ability of the chicks to gain weight, on a per Calorie basis, was independent of the amount and kinds of fat in the diet.

A close correlation between weight gain and total caloric intake (feed consumption times productive energy of the diet) was observed for each of the three treatments. Total caloric intake, highest for chicks in treatment 3, may account for the greater weight gains which differed significantly from those in treatments 1 and 2.

Blood taken from 10 chicks on the first day after hatching showed an average total serum cholesterol concentration of 500 mg/100 ml. When determinations were made on blood samples of individual one-week old chicks, average total serum cholesterol concentrations in each of the three treatments had dropped sharply (Figure 1) and continued to decline rapidly during the next two weeks. Figure 1 also shows a similar serum cholesterol pattern in a preliminary study made by this investigator of a group of 25 chicks fed a stock diet containing 3% soybean oil. Average total serum cholesterol level was lower in the

Figure 1

TOTAL SERUM CHOLESTEROL CONCENTRATIONS OF CHICKS FED VARIOUS EXPERIMENTAL DIETS



one-week old chicks than for any of the groups in the present experiment, and concentrations declined more gradually from the first to the fourth weeks. In this preliminary study cholesterol determinations were not taken at the time of hatching.

These observations on the cholesterol levels of chicks during the first few weeks after hatching are similar to those reported by several investigators. Entenman et al. (1940), observed a rapid drop in blood cholesterol during the first week with a gradual decline for a remaining 36-day experimental period. Rodbard et al. (1951) and Cherms et al. (1960) reported that cholesterol level of chicks fed ordinary mash diets dropped during the first week to about one-third the level at hatching. They observed no significant reductions in serum cholesterol during the remaining experimental weeks.

Weekly mean total serum cholesterol concentrations of chicks in each dietary treatment are presented in Table 5. Lowest means were observed in chicks receiving 10% safflower oil, and highest in those fed the "fat free" rations.

Analysis of variance (Table 5a) indicated significant differences in serum cholesterol among groups due to dietary treatment. Chicks in treatment 3, from three to eight weeks of age, exhibited mean total serum cholesterol values significantly different from treatments 1 and 2, but there were no significant differences between 1 and 2 except at five weeks of age.

Correlation coefficients determined for each treatment for cholesterol, body weight, feed efficiency, caloric intake, and weight gain did not differ significantly among the three treatments. Any

Table 5

MEAN TOTAL SERUM CHOLESTEROL, mg/100 ml, OF CHICKS ONE TO EIGHT WEEKS OF AGE*

Treatment	Weeks on Diet								
	1	2	3	4	5	6	7	8	All Weeks
1	271.7	204.6	181.1[1]	179.1[1]	200.7[1]	166.7[1]	170.6[1]	170.2[1]	191.6[1]
2	255.5	185.9	171.0[1]	170.0[1]	185.2[2]	163.6[1]	170.8[1]	169.9[1]	184.2[1]
3	246.8	186.4	154.2[2]	158.6[2]	153.7[3]	128.0[2]	143.1[2]	150.8[2]	166.9[2]
±SE	(ns)	(ns)	4.27	3.27	3.30	3.96	3.27	4.30	2.96

*Significant difference is indicated by number superscripts. Any means followed by the same or no superscripts are not significantly different ($P < 0.05$).

Table 5a

ANALYSIS OF VARIANCE (MEAN SQUARES)

Source of Variation	df	Weeks on Diet 1	2	3	4	5	6	7	8	All Weeks
Replicates	1	4.10	3.99	137.21	10.37	8.97	51.07	135.50	1791.43	.28
Diets	2	320.94	227.07	367.02*	211.23*	1147.19*	923.51*	505.89*	247.28*	321.50*
R X D	2	135.04	426.60	91.20	125.90	23.56	30.70	10.89	23.10	11.39
Among Chicks	‡	155.06	91.54	38.76	22.93	23.20	33.12	22.57	38.94	18.75

*Significant difference ($P < 0.05$).

‡Due to losses of blood samples, number varied slightly, with df within pens ranging from 51 to 59.

relationship existing between any of these measures within a treatment, therefore, was not due to the level or type of dietary fat.

A high inverse relationship between serum cholesterol level and weight gain of chicks is observed in all treatments. Correlations which do not differ significantly among treatments may indicate that the greater decrease in serum cholesterol in treatment 3 is associated with the greater gain in weight of this group. In a study with humans, Ahrens et al. (1954) observed that higher serum cholesterol levels were associated with gain in weight. Bumgardner (1955) reported a similar finding in four-week old chicks. He suggested that higher cholesterol levels found in male rather than female chicks were due to greater body weight. Cherms (1960) and Hardy (1961) found no relationships between growth rate, feed conversion, and serum cholesterol in chicks.

The present experiment is in agreement with previous reports on man and animals in which diets containing highly unsaturated vegetable oils produce lower serum cholesterol concentrations than diets containing more saturated oils or hydrogenated fats.

March and Biely (1959) observed in the chick that when endogenous cholesterol is responsible for the major part of circulating cholesterol, the effect of fat is related predominantly to removal of cholesterol. Highly unsaturated corn oil produced lower serum cholesterol levels than more saturated hydrogenated cottonseed oil when fed to three and five-week old chicks on cholesterol-free diets with protein levels at 20 to 26%.

Entenman et al. (1940) reported that the chick emerges from the egg with large deposits of lipids in the liver, yolk sac, and blood. The liver contains enormous amounts of cholesterol, the major part of which is in the esterified form. The yolk which nourishes the chick before hatching contains large amounts of both fat and cholesterol. These workers proposed that the cholesterol in the yolk esterifies with the fatty acids for transport and storage in the liver. The cholesterol store is more rapidly removed from the liver than are the fatty acids which form triglycerides resulting in the fatty liver observed at hatching. The high cholesterol ester concentration of the blood at hatching is due to the enclosure of the yolk within the abdominal wall just prior to hatching and then absorption into the blood stream. Removal of the cholesterol esters from the yolk sac normally proceeds at a slower rate than removal of neutral fat and other lipids.

These observations, then, may suggest a possible mechanism for the more rapid lowering of serum cholesterol levels in chicks fed safflower oil as compared to those on either the partially hydrogenated fat or "fat free" diets. The high polyunsaturated fatty acid content of the safflower oil may accelerate cholesterol transport to the liver as well as increase metabolism in the liver. This may be due to the preferential esterification of cholesterol with the polyunsaturated fatty acids which numerous workers have observed to occur.

The effects of the three dietary treatments on serum proteins and lipoproteins are shown in Tables 6 and 7, respectively. Electrophoretic patterns for both protein and lipoprotein fractions varied little within treatments during the four-week period.

Table 6

SERUM ALBUMEN TO GLOBULIN RATIOS OF CHICKS FIVE TO EIGHT WEEKS OF AGE

Treatment	Chick No.	Weeks 5	6	7	8	Average
1	4	1.66	1.66	1.79	1.68	1.70
2	22	1.49	1.79	1.50	1.62	1.60
3	24	1.27	1.39	1.26	1.28	1.30

Table 7

SERUM BETA- TO ALPHA-LIPOPROTEIN RATIOS OF CHICKS FIVE TO EIGHT WEEKS OF AGE

Treatment	Chick No.	Weeks 5	6	7	8	Average
1	4	0.22	0.23	0.26	0.32	0.26
2	22	0.15	0.23	0.21	0.26	0.21
3	24	0.24	0.21	0.24	0.31	0.25

The albumin to globulin (A/G) ratios were lowest for the chick receiving safflower oil, however, they varied little during the four weeks and showed no relationship to changes in serum cholesterol concentrations. It may be assumed, therefore, that the lower ratios for this chick were due to individual variation. Little difference was observed between the A/G ratios of chicks in treatment 1 and 2. Leveille et al. (1960) observed that dietary fat and dietary cholesterol have little influence on serum proteins of the chick, but when dietary protein level is increased serum albumin level is markedly increased. A/G ratios of chicks in treatments 1 and 2 agreed closely with those observed by Leveille and Sauberlick (1961) in chicks fed dietary protein at the 20 to 25% level.

The ratios of beta- to alpha-lipoproteins agreed closely among chicks in the three treatments and showed no relationship to serum cholesterol or to amount and kinds of fat in the diet.

CHAPTER IV

SUMMARY AND CONCLUSION

Day-old cockerels were placed on three experimental diets, namely, (1) a "fat-free", cholesterol-free diet, (2) the same diet with 10% by weight of partially hydrogenated safflower oil substituted for an equal weight of cerelose, (3) diet 2 repeated, but with addition of safflower oil instead of the hydrogenated product.

Weight gains, feed consumption, feed efficiency, calories required per gram gain in weight, and serum cholesterol values were obtained for 22 chicks in each dietary treatment for a period of eight weeks. Electrophoretic protein and lipoprotein patterns of serum from one chick per treatment were obtained weekly from the fifth to the eighth week.

Total serum cholesterol values were lowest in chicks on the safflower oil diet. Values differed significantly ($P \leqslant 0.05$) from those obtained in chicks in the other two treatments from the third to eighth week of the experiment. Cholesterol values for chicks on the partially hydrogenated fat diet were slightly lower than for those on the "fat-free" ration, but significant differences were observed for only the fifth week.

No significant differences were observed among treatments in the ability of the chicks to gain weight on a per Calorie basis. This

indicated that the chicks were as able to absorb and metabolize, for growth, the partially hydrogenated safflower oil (high in trans-oleic acid) as the natural oil (high in linoleic acid). Within treatment correlations among serum cholesterol, body weight, feed efficiency, feed consumption, and weight gain did not differ significantly among the three treatments, indicating that any relationship found between any of these factors was not due to the amount and kinds of fat in the diets. Lower serum cholesterol levels were observed to be associated with greater increases in body weight.

The albumin to globulin and beta- to alpha-lipoprotein weekly ratios showed little difference among treatments and did not appear to be affected by the amount and kinds of dietary fat. No significant relationship could be observed between these ratios and the respective serum cholesterol concentrations for any of the treatments.

The results obtained under these experimental conditions indicate that the amount and kinds of fat in a cholesterol-free diet of the chick influence primarily the level of total serum cholesterol. The natural and partially hydrogenated safflower oil used in the high fat diets differed widely in P/S ratio, iodine value, and the proportion of cis- to trans-acids, but total chain lengths were approximately the same. Since serum cholesterol values for the "fat free" and partially hydrogenated safflower oil diets were closely similar, and higher than for the oil, the conclusion may be made that the factor in the safflower oil which produced lower cholesterol concentrations was the high content of polyunsaturated linoleic acid. A possible mechanism

for removal of cholesterol from the circulation of the chick has been suggested, based on relationship of polyunsaturated fatty acids to accelerated transport and metabolism of cholesterol.

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A P P E N D I X

I. FRACTIONATION OF SERUM PROTEINS

Refer to the instructions for the operation of the Spinco Model R Paper Electrophoresis System equipment in following method for determination of lipoproteins when noted.

Preparation of B-4 bromophenol blue dye. Pour one bottle of dye into a 1000 ml volumetric flask using a funnel. Rinse the dye from the cap and the bottle four times with cold methanol, then bring to volume with additional cold methanol. The dye may be used several times. If volume decreases through evaporation, add more cold methanol.

Preparation of buffer and Versatol standard. Follow instructions as given for lipoproteins.

Preparation of 5% acetic acid solution. Fill a 2 liter volumetric flask one-half full with redistilled water. Add 100 ml glacial acetic acid and bring to volume with redistilled water.

Operation of cell. Follow instructions as given for lipoproteins with the following exceptions: (1) apply 6 µl of serum to paper strips with one application per strip, (2) for electrophoresis, turn the output adjust switch to 2½ ma but use the same ammeter and constant current settings as for lipoproteins, (3) proceed with electrophoresis at room temperature. Place the cell out of drafts, at least eight inches away from the Duostat, and out of the path of direct light or heat.

Treatment of strips after electrophoresis. To dry strips, remove light bulb from oven and set the temperature at 130 C. Preheat the oven for 15 minutes. Place the strips in the oven on a perforated rack. Turn off the heat and allow the strips to dry for 30 minutes. Record the maximum and minimum drying temperatures.

Transfer the dried strips to the staining and rinsing rack. Immerse the rack in reagent grade methyl alcohol for 10 minutes. Then immerse strips in bromophenol blue dye for 30 minutes.

Rinse the strips for six minutes in three separate 5% acetic acid solutions. Place on perforated rack, and dry for 15 minutes in oven which has been preheated for 15 minutes at 130 C.

Remove strips from the staining and drying rack and place in a dessicator with a small open jar of liquid ammonium hydroxide for at least 15 minutes. Remove strips, one at a time, for scanning.

Scanning of strips. Follow instructions as given for lipoproteins except use a calibration set-point of 8.2. Set the recording pen to 8.2 cm above the zero line on the chart.

Analytrol pens. Follow instructions as given for lipoproteins.

Cleaning of cell. Follow instructions as given for lipoproteins.

Cleaning of glassware. Follow instructions as given for lipoproteins.

II. FRACTIONATION OF SERUM LIPOPROTEINS

The Spinco Model R Paper Electrophoresis System for separation and photometric scanning of lipoprotein fractions includes: Durrum-type paper electrophoresis cell, Duostat power regulator, and Analytrol (a calibrated recording densitometer and automatic integrator).

The staining method as described below produces a clear background and makes possible the recognition of six lipoprotein fractions, which correspond to their respective proteins.

Preparation of stock Ciba fat red 7 B dye. Place 0.5 g of dye in a 1000 ml volumetric, and bring to volume with reagent grade, absolute ethyl alcohol. Let stand two days, filter, and dilute in 667 ml redistilled water. Cap with parafilm and let stand for 24 hours. Remove excess dye by double filtration. Use dye once only. Since increase in temperature causes more dye "pick up", record room temperature changes during use of the dye.

Preparation of buffer. Carefully cut across the very top of the package of barbital buffer (B-2, Spinco No. 300-824) and empty contents into a transfer funnel placed in a 1000 ml volumetric. Rinse the funnel and buffer package five times with redistilled water. Add additional redistilled water to fill volumetric flask almost to the neck. Swirl contents to mix well. Bring to volume and mix by inverting flask several times.

Preparation of Versatol standard. Dilute Versatol with 5 ml redistilled water and seal bottle with tape. Standard will keep one week in the refrigerator.

Preparation of bleach (0.1 % Chlorox in 2% acetic acid). To prepare a 2% acetic acid solution, fill a 2 liter volumetric flask one-half full with redistilled water. Add 40 ml glacial acetic acid, and bring to 2000 ml volume with redistilled water.

Pour approximately 25 ml of the acetic acid solution into a 250 ml beaker. Add 2 ml of Chlorox to the remaining solution in the volumetric. Bring to 2000 ml volume with the acetic acid solution in the beaker.

Operation of cell. To set up the cell, insert the four baffles, two wick supports, and the wicks. Number and date eight paper strips (Spinco No. 300-846) and include all pertinent data. Place the support stand on the cell so that the slit in each leg fits over the center partition and into the grooves. Place the extended folding rack on the support stand and swing the legs of the folding rack down as far as they will go. Arrange strips on the rack with number one at the right. Mark the strips with a 4H pencil at the center to indicate the point or origin of serum application. Do not touch strips unnecessarily.

Introduce the buffer, using one liter of the buffer solution previously prepared. Pour approximately 800 ml of the solution into the cell vessel and replace the cell cover. Using a plastic funnel, pour the remaining buffer over the top of the paper strips (run the

funnel back and forth along the cover slot to wet all strips). Seal the cover-slot with electrical tape. See that each paper strip is in contact with the paper wick in a straight line.

To equalize the fluid levels, raise the end of the cell opposite the plugs, one to two inches for 15 seconds. See that fluid level is 1/16 inch above the top of the fluid baffles and 1/16 inch below the top of the removable baffles.

Allow 15 minutes for the excess liquid to drain from the strips and for the closed cell to become saturated. Seal the entire cell with electrical tape.

Apply the serum in subdued light. Fill a micropipette with 10 μl of serum and transfer to the sample applicator. Use a clean pipette for each sample. Apply the serum to the paper strip by resting the applicator on the middle of the glass support rod, being careful to center the applicator over the paper strip. Depress the applicator briskly for five seconds to insure covering the entire width of the strip. Make two 10 μl applications of serum per strip, sealing the slot between applications.

Connect the Duostat by inserting one of the connector plugs attached to the back of the Duostat into the plugs at the end of the cell. The unused plug must be connected with the pair of plugs on the back of the Duostat to complete the safety interlock circuit.

To begin electrophoresis, turn the output adjust switch to the right and allow to warm up. Adjust until meter indicates current is 4 ma. Set ammeter at 0-15 ma, and the constant current at 2-30 ma. Proceed with electrophoresis for 17 hours with the cell refrigerated at

4-10 C. during operation. Record the time and temperature and set the maximum-minimum thermometer.

To terminate electrophoresis, turn the output adjust switch on the Duostat to "off." Remove the connector plug from the cell and take off the cell cover. Immediately remove the paper strips from contact with the wicks. Open the rack to the extended position, tighten the thumb nuts, and remove the rack from the support stand. Replace the cell cover, and record the time and temperature.

Treatment of strips after electrophoresis. Place strips in a drying oven for seven minutes at 100-110 C. Transfer the dried strips to the staining and rinsing rack. Immerse the rack in an enameled sterilization tray containing fat red 7 B dye for one hour at room temperature. Then, immerse the strips in the bleach solution for approximately one minute 15 seconds. Rinse the strips for 15 minutes in each of four separate 2% acetic acid solutions. Place on blotting paper with a second sheet of blotting paper on top, and dry on the folding rack at room temperature out of direct light.

Scanning of strips. Connect the Analytrol to the wall outlet and turn on the transformer which is set at 115. Turn on the power and lamp switches and allow to warm up for at least 15 minutes. Center the slit and use as set at 2 mm.

Lift the pen guard and fill the pens with two to three drops of ink using a dropper. The ink should flow freely.

Raise the strip engaging lever and insert the paper strip with the pattern-free region in front of the slit in the front aperture.

Lower the engaging lever.

Pull the pen lift-button and insert the chart, squared and flush against the front margin of the instrument. The narrow strip of ruled lines at the bottom of the chart should be in place at the front of the instrument. Press the pen lift button.

Turn on the pen and feed switches and leave until several centimeters of the end of the paper strip which contains zero concentration have been scanned. Turn off the feed switch and examine the tracing to see if the average displacement coincides with the zero line. If the average displacement does not coincide, estimate the distance that it lies above or below the zero line.

To calibrate, turn the calibration-filter knob fully counter clockwise which places the light region of the calibrated neutral density filter in the light path. Set the recording pen to the zero line by turning the zero adjust knob. If the pen does not come to the zero position, check that the potentiometer knob is near the middle of its range. Put the dark region of the calibrated neutral density filter in the light path by turning the calibration-filter knob fully clockwise. Find the calibration set-point as recorded on the inside of the raised pen guard which is 8.5 on the instrument. Turn the cell potentiometer knob to set the recording pen to 8.5. This is 8.5 cm above the zero line on the chart. Since the zero and filter adjustments affect one another, it may be necessary to repeat the calibration procedure one or more times. Return the calibration-filter knob to fully counter-clockwise position to bring the recording pen back to the zero line on the chart.

Scan the pattern. Then, turn off the feed and pen switches and remove the chart. Swing the strip-engaging lever up to unclamp and remove the paper strip.

Repeat the procedure for each successive scanning. When all strips have been scanned, turn off all switches and clean the pens.

Analytrol pens. Parker 51 ink is preferred for use in the pens since it is black and makes a clearer outline than the blue ink supplied by Spinco. Remove the pen from the wooden holder when filling, and press the ink through the point with finger.

After using, remove the pen immediately and rinse in distilled water, being careful to protect the points while washing. Do not soak the pens, especially while in the wooden holders, because they will warp. Force distilled water through the pens holding a finger on top until clear water flows through. It may be necessary to use suction to remove all of the water. Dry the pens for about one hour before replacing on the instrument.

Cleaning of cell. Rinse the cell with tap water, and then remove partitions and wash all parts in warm water and detergent (Vel). Do not immerse the electric outlet in water. Use a small soft test tube brush to remove crystals from the electrode wires being careful to avoid damaging the wires.

Rinse the cell and partitions eight times in each of the following: tap water, distilled water, and redistilled water. Dry the parts and exterior of the cell with paper towels, and invert the cell on a drying rack. Cover the parts to avoid dust. The cell should be

empty and dry when not in use. Keep the cell away from cleaning solution and high temperatures.

Cleaning of glassware. Rinse eight times with tap water. Place in potassium dichromate cleaning solution for at least one hour. (Calibrated glassware should remain in the cleaning solution no longer than one hour since it will be etched if kept in the solution for prolonged periods.) Drain off excess cleaning solution which may be reused several times. Rinse the glassware eight times in each of the following and in the order given: tap water, distilled water, and redistilled water. Dry the exterior of the glassware with paper towels, and invert on clean paper towels to dry.

To clean pipettes, rinse immediately with tap water and leave in water until all samples have been applied and the cell is in operation. Place in potassium dichromate cleaning solution for at least one hour. Using water suction, rinse pipettes eight times in each of the following: tap water, distilled water, and redistilled water. Air dry with water suction and place in drying oven for 15 minutes to complete drying.