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Studies on the Analysis and Esterification of Cholesterol

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STUDIES ON THE ANALYSIS AND ESTERIFICATION OF CHOLESTEROL

BY

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This thesis is approved as a creditable, independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree; but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

The recent widespread interest in the cause of atherosclerosis in humans has stimulated research in many fields of science. Articles describing cholesterol experiments on humans and lower animals indicate that an elevation of the blood level of cholesterol increases the incidence of atherosclerosis (1, 2, 3, 4, 5). Bloor and colleagues (6, 7) suggested an interrelationship between the metabolism of cholesterol and the highly unsaturated fatty acids of the blood. In these experiments it was found that the fatty acids present in neutral fat had the lowest unsaturation, the phospholipids contained the fatty acids of intermediate unsaturation, and the fatty acids present in cholesterol esters had the highest unsaturation. Alfin-Slater *et al.* (8) in 1954 studied the effect of fatty acid deficiency in rats. They showed that the cholesterol content of the liver increased with diets of low fatty acid content and that the increase was confined almost exclusively to the ester fraction. They suggested that the absence of the essential fatty acids resulted in increased levels of cholesterol in the liver, the adrenal glands, and the blood. They further postulated that this increase may have been the result of cholesterol esters containing saturated fatty acids. This particular cholesterol ester may not be easily metabolized.

Statistical analysis of per capita fat consumption (9) and experiments dealing with the type of fat consumed (10, 11) indicate that a high consumption of fat containing mostly saturated fatty acids increases the incidence of atherosclerosis. It has also been shown

that a diet high in unsaturated fatty acids tends to lower the blood level of cholesterol. This evidence stimulated much interest in the biochemistry of the essential fatty acids.

Because the fatty acids of the cholesterol esters are primarily the unsaturated ones and the unsaturated fatty acids are inducive to a lowering of the serum cholesterol level, it was of interest to study the mechanism controlling the formation of these esters. The function of the cholesterol esters, their role in atherosclerosis, and the part they play in the transport and metabolism of each lipid constituent are, at this time, not clear.

LITERATURE REVIEW

The enzyme cholesterol esterase appeared in the literature as far back as 1910 when Kondo (12) reported that extracts of horse liver and ox liver catalysed the hydrolysis of cholesterol esters. Cholesterol esterase also aroused interest when Mueller (13) in 1915 found that the ingested free cholesterol could be removed in the thoracic chyle as cholesterol esters. Schoenheimer et al. (14, 15) thought it possible that a specific cholesterol esterase was responsible for the high selectivity of sterol absorption. Their experiments demonstrated that, while cholesterol is readily absorbed, other sterols are absorbed only in traces. The presence of such a highly specific cholesterol esterase in subcutaneous tissue was also indicated by experiments of Schoenheimer and Yuasa (16), who showed that cholesterol placed under the skin is esterified, while non-absorbable sterols remain unchanged.

Yamamoto, Goldstein and Treadwell (17), Swell and Treadwell et al. (18, 19, 20, 21, 22, 23, 24), and Sperry and Brand (25) have contributed much to the study of the chemical and physical requirements for optimum activity of cholesterol esterase and to its classification. One of the better summaries of the optimum requirements for cholesterol esterase appears in a more recent publication by Swell and Treadwell (20). In this article they list the optimum pH and activity for hydrolysis and esterification of cholesterol esters and cholesterol respectively with thirteen different acids, from acetic acid to oleic acid inclusive. In their experiments they used pancreatin as the

source of the enzyme. The rate of esterification of cholesterol with oleic acid was much greater than the rate of esterification of cholesterol with acetic acid. A great difference in the rate of esterification was noted between caproic acid and caprylic acid and also between the stearic acid and oleic acid. The optimum pH for esterification of cholesterol with oleic acid was higher (0.6 pH units) than the optimum pH for any of the other acids listed. In this article they also reported the influence of different emulsifying agents on the activity of the enzyme.

Cholesterol esterase from other sources has also been studied but not with such a large number of fatty acids. The enzyme from rat blood was studied by Sperry and Stoyanoff (26), by Sperry (27) and by Swell and Treadwell (19). Rat blood did not appear to be a very good enzyme source because of the presence of buffers, free and esterified cholesterol, emulsifying agents and many other constituents in the blood or serum. The rat liver has been used as a source of the enzyme by Sperry and Brand (25), Byron, Wood and Treadwell (28), and by Nieft and Duel (29). Sperry and Brand (26) also studied the cholesterol esterase present in rat brain.

The proof that the active agent is an enzyme appears in several publications (15, 30). Fodor (30) subjected the enzyme to heat, to varying hydrogen ion concentrations, and to crystalline trypsin. In testing the activity, he found that heating the enzyme to 60 degrees centigrade for twenty-five minutes, pretreating the enzyme to a pH of ten, and pretreating with crystalline trypsin inactivated the enzyme. Swell and Treadwell (21) also noted that temperatures around 60 to 65

degrees centigrade inactivated the enzyme. They also studied the effect of varying cholesterol and enzyme concentrations upon the rate of esterification.

Many studies have been conducted with the enzyme cholesterol esterase using many different acids; however, no study of cholesterol esterification with the essential fatty acids has been noted. Because of the importance of the essential fatty acids in atherosclerosis, it seemed of interest to study the optimum pH for the enzyme, using the essential fatty acids as substrates.

ANALYSIS OF CHOLESTEROL

The first objective in the study of cholesterol esterase should be to obtain a reliable method of analysis for the total and free cholesterol in the material under investigation. Because of the many discrepancies in the results presented in papers on cholesterol esterase, accuracy in the methods for determining total and free cholesterol have been questioned.

THE SPERRY AND WEBB METHOD

Experimental

One of the most widely accepted and used methods for analysis is a revision of the Schoenheimer and Sperry method (31) by Sperry and Webb (32). The procedure is reviewed here for the purpose of clarity.

Extraction of Cholesterol from Blood Serum (or other material):

> About 2 milliliters of an acetone and ethanol mixture (1:1) are placed in a 5 milliliter volumetric flask, and 0.2 milliliters of serum is added slowly in such a manner that it runs down the wall of the flask and forms a layer under the solvent. As soon as the pipette is withdrawn, the contents are mixed thoroughly by a swirling motion. A finely divided precipitate should result. The solvent is brought just to a boil in the steam bath with agitation to prevent bumping, the flask is cooled, acetone and ethanol mixture (above) is added to the mark, and the suspension is thoroughly mixed and filtered into a small test tube. Aliquots of the clear filtrate for free and total cholesterol determinations are pipetted at once to avoid evaporation.

Precipitation of Free Cholesterol:

> To 2 milliliters of the filtrate in a centrifuge tube 1 drop of 10 per cent acetic acid solution and 1 milliliter of 0.5 per cent digitonin in 50 per cent ethanol are added. The contents are stirred thoroughly

with a rod which is left in the tube. The tube is placed in a preserving jar which is covered tightly and left overnight at room temperature.

The tube is transferred to a rack, the contents are stirred gently to free any precipitate which may adhere to the wall near the surface of the liquid and the rod is removed without contact with the upperpart of the tube and placed on a rack made of heavy wire and so designed that a number of rods may be held without danger of rubbing off the adherent precipitate. The position of the rod is noted so that it may be returned to the proper tube. The tube is centrifuged for 15 minutes at 2,800 revolutions per minute and the centrifuge, which should be clear, except for the occasional presence of a few particles floating at the surface, is decanted with a slow, steady motion and watched closely in a good light as the fluid leaves the precipitate. If any precipitate is seen to be suspended, the sample is recentriuged, or better, discarded. If this happens frequently, the time or speed of centrifuging should be increased. The tube is drained for a few moments and the last drop is removed by touching the lip to a clean towel. The rod is returned to the tube, the wall of the tube and the rod are washed down with 1.5 to 2 milliliters of an acetone and ether mixture (1:2), the contents are stirred thoroughly, the rod is returned to the rack, the tube is centrifuged for 5 minutes, and the centrifugate is decanted. The precipitate is washed twice more in the same manner with ether. The rod is returned to the tube which may be stored for several days at this stage. If color development is to follow at once, the ether is evaporated by placing the tube for a few minutes in a moderately warm water bath.

Precipitation of Total Cholesterol:

One drop of potassium hydroxide solution (10 grams of pure potassium hydroxide dissolved in 20 milliliters of water) is placed in a dry centrifuge tube, 1 milliliter of the serum extract is added, and the mixture is stirred with a vigorous up and down motion of a rod until no droplets of the alkali solution can be seen at the tip of the tube. A preserving jar containing a layer of sand about 3 centimeters deep is heated in a water bath until the temperature of the sand is 45 degrees centigrade. The tube is placed in the sand, and the jar is covered tightly and placed in an incubator at about 3 degrees centigrade for 30 minutes.

The tube is removed to a rack and cooled. The stirring rod is raised, acetone-ethanol (1:1) is added to the 2 milliliter mark, and the alkali is neutralized to the phenolphthalein end-point with 10 per cent acetic acid. From 4 to 6 drops should be required if the drop of alkali solution was of proper size. An extra drop of acetic acid and 1 milliliter of digitionin (above) solution are added and the sample is treated as described for free cholesterol, except that the precipitate is washed with ether only once.

Development and Reading of Color:

The tubes are placed in order of reading in a sand bath at 110 to 115 degrees centigrade in an oven for 30 minutes. The temperature of a water bath in a dark cabinet is adjusted to 25 degrees centigrade and maintained there during the rest of the procedure by the addition of hot or cold water as needed. The sand bath is removed from the oven, and 1 milliliter of pure glacial acetic acid is added to the first tube while it is still in the hot sand. The contents are stirred vigorously, and the tube is left in the sand while the acid is being added to the next two or three tubes; 2 or 3 minutes in all. The solution is stirred again, and the tube is removed from the sand, cooled, and placed in the water bath. The process is continued until all the tubes are in the water bath in order of reading. A tube containing 1 milliliter of standard solution (0.1 milligram of cholesterol per 1 milliliter) is placed at the beginning of the series of tubes and another at the end.

An amount of acetic anhydride (99 to 100 per cent) sufficient for the number of samples to be read is placed in a glass stoppered flask and chilled in an ice bath. With the flask still in the ice bath, concentrated sulfuric acid is added in the proportion of 1 milliliter to 20 milliliters of acetic anhydride with agitation during the addition. The stopper is inserted, and the flask is removed from the bath, shaken vigorously for a few minutes, and returned to the bath. A blank containing 1 milliliter of acetic acid and 2 milliliter of the reagent is prepared.

About 10 minutes later, when the reagent is thoroughly chilled, the first tube is removed from the 25 degree water bath and wiped dry, 2 milliliters of the cold acetic anhydride-sulfuric acid reagent are added, the contents are stirred vigorously, the rod is removed, and the tube is returned to the bath. If the reading is to be carried out in a photometer which uses matched cuvettes of the

test tube type, the solution is poured at this point into a cuvette, which is placed in the series at time intervals best controlled by a stop-watch, such that the time of color development before reading is the same for all samples. Although the color is fairly stable at its maximum from 27 to 37 minutes after the acetic anhydride-sulfuric acid solution is added, it is best to read all samples after as nearly the same time of color development as possible, preferably 30 to 31 minutes. The temperature of the water bath is noted, and adjusted, if necessary at frequent intervals during the color development and reading.

The changes that were made in the above procedure were as follows: In the extraction of the substrate (or blood serum) one milliliter of substrate was diluted to 25 milliliters with acetone-ethanol in place of the 0.2 milliliters of substrate and five milliliters volume. To eliminate rapid evaporation of the solvent, creating concentration of the cholesterol during filtration, a watch glass was placed over the funnel immediately after filling it with the material to be filtered. The rest of the procedure was followed as outlined.

A calibration curve was made using recrystallized cholesterol dissolved in glacial acetic acid. After adding the acetic anhydride-sulfuric acid reagent to a series of cuvettes containing one milliliter of a specified cholesterol standard and allowing the color to develop as prescribed, the per cent transmittance was obtained by using both a Beckman DU spectrophotometer and a Bausch and Lomb "Spectronic 20" spectrophotometer.

The first esterification determination was made using palmitic acid and recrystallized cholesterol as the substrates. Four substrates were prepared as suggested by Sprell and Treadwell (21): 12.5 milligrams of palmitic acid, 0.5 milliliters of ether, 4.5

milliliters of 0.125 molar phosphate buffer at a 5.2 pH, 0.5 milliliters of a 10 per cent solution of sodium taurocholate, 0.5 milliliters of a 1:1000 solution of merthiolate, and 250 milligrams of impalpable dried egg albumin were homogenized for two minutes in a Potter-Elvehjem homogenizer. The solution was then placed in a Dubnoff Metabolic Shaker, which was adjusted previously to a temperature of 37 degrees centigrade for one hour to remove the ether. This homogenate was shaken at 120 oscillations per minute.

The enzyme extract was prepared as follows: A white adult rat was decapitated and the liver immediately removed and placed on ice. A weighed amount of liver was homogenized in the Potter-Elvehjem homogenizer with a 1:1 dilution of glycerin and water so that a 20 per cent homogenate of the liver resulted. This was then transferred to a centrifuge tube which was then corked and placed on a revolving vertical wheel for fifty minutes to facilitate extraction of the enzyme. It was then centrifuged for 5 minutes at 2500 revolutions per minute in a number 1 National centrifuge.

One milliliter of the enzyme extract was then placed in the above substrate, quickly mixed, and 1 milliliter removed for cholesterol extraction with alcohol-acetone. This constituted the zero hour sample. This was prepared in duplicate. Two control samples were analyzed using 1 milliliter of water-glycerin in place of the enzyme extract. The four preparations were placed in a Dubnoff Metabolic Shaker which was previously adjusted to 37 degrees centigrade. These preparations were agitated at the rate of 120 cycles per minute for

18 hours. At the end of the incubation period another 1 milliliter aliquot was removed for cholesterol extraction. The results obtained using the method of analysis previously described are given in Table I.

Results and Discussion

Because of the discrepancies in the results, the procedure was repeated; however, the results obtained from the second determination had similar discrepancies. From Table I it is obvious that either some of the cholesterol is lost in the total cholesterol analysis or the free cholesterol procedure gives misleadingly high results. A check on the accuracy of the analysis was indicated. Another standard curve was made using a freshly prepared standard solution of cholesterol in acetic acid. The resultant standardization curve showed no significant change. As a check on the method an attempt was made to get 100 per cent recovery of cholesterol dissolved in alcohol-acetone. To accomplish this a series of solutions of known cholesterol concentration were prepared and each treated as though it were a filtrate from the extraction step of the analysis. The total and free cholesterol portion of the method was run, and the results are indicated in Table II.

This method of checking the procedure was repeated, and data similar to those found in Table II were obtained. From Table II it is readily noted that less than 100 per cent of the cholesterol is recovered with the total cholesterol portion of the Sperry-Webb method. Instead of working further with this method, it was decided to try

TABLE I. CHOLESTEROL VALUES OBTAINED FROM AN ESTERIFICATION DETERMINATION USING THE SPERRY-WEBB METHOD OF ANALYSIS.

Sample Number		Cholesterol Before Incubation		Cholesterol After Incubation	
		Free	Total	Free	Total
1	Without enzyme	1.98*	1.50	1.85	1.84
2	Without enzyme	1.75	1.72	1.87	1.74
3	With enzyme	1.98	1.86	1.87	1.84
4	With enzyme	2.40	1.16	2.40	1.16

* The values are expressed in milligrams per milliliter of substrate.

TABLE II. CHOLESTEROL RECOVERY USING THE SPERRY-WEBB METHOD OF ANALYSIS

Alcohol-Acetone Standard Number	Mg. Cholesterol per ml. of Standard	Mg. Recovered	
		Total	Free
1a	1.00	0.77	
1b	1.00	0.72	
2a	1.50	1.27	1.48
2b	1.50	1.05	
3a	2.00	1.61	1.98
3b	2.00	1.52	
4a	2.50	2.02	2.50

another method of analysis.

THE WYCOFF AND PARSONS METHOD

Experimental

An ingenious method of separating the esters of cholesterol from free cholesterol with the use of a silicic acid column was reported by Wycoff and Parsons (33). These authors recommended either the use of 100 mesh silicic acid sold by Mallinckrodt especially for column chromatography, or the activating of ordinary reagent grade products by heating them at 120 degrees centigrade overnight and quickly sieving them once in a moist atmosphere. The activated silicic acid must be protected from moisture in the atmosphere. Their procedure follows:

> A glass tube about 25 centimeters long with an 8 millimeter outside diameter is used for the chromatographic tube. The tube is constricted to a diameter of 2 to 3 milliliters inside, on one end and drawn out about 10 millimeters. The column is prepared by ramming a small amount of cotton down the tube to plug the constricted end. The silicic acid is then placed in the tube to a height of about 5 centimeters by means of an eye dropper and packed by applying air at a pressure of 7 to 10 pounds per square inch to the top of the column. Two milliliters of petroleum ether or Skelly Solve B is then forced through the column with the same air pressure.
>
> For the routine assay of human plasma or serum (or other material to be extracted), the procedure is as follows:
>
> Mix 0.1 milliliter of material to be extracted with 5 to 7 milliliter of 1:1 methanol and acetone. Bring the mixture to boiling, cool, dilute to 10 milliliters with solvent, and filter. Evaporate a 3 milliliter aliquot of the filtrate in a small tube by placing the tube in cold water and bringing the water to boiling. Invert the tube while it is cooling to drain the residual vapor of the solvent. Transfer the lipid to the column with three 2

milliliter portions of petroleum ether by means of an eye dropper.

Rinse the test tube and the chromatographic tube with each of the 2 milliliter portions of petroleum ether and force each through in turn. The flow rate should never be greater than 4 milliliters per minute with any of the solvents. Care should also be taken never to allow the solvent level to go below the top of the silicic acid in this step and in the next one. At this point the free and esterified cholesterol are adsorbed at the top of the column.

Develop the column with about 4 milliliters of 1:1 chloroform and petroleum ether. Collect this eluate, which contains the esterified cholesterol, in a separate test tube. Rinse the outside of the tip of the chromatographic tube with about 1 milliliter of petroleum ether, allowing the ether to run into the same test tube. To remove the free cholesterol from the column elute with 4 milliliters of 1:1 methyl or ethyl acetate and petroleum ether. Force this through until flow ceases, and rinse the tip as before. Extrude the column by means of air pressure and discard the silicic acid.

Evaporate the solvents as before by placing the tubes in cold water and bringing the water to a boil. Take the fractions up in 3 milliliters of glacial acetic acid. Warm the tubes containing the esters to effect solution. Cool and add 2 milliliters of the ferric chloride—sulfuric acid solution of Brown (34) and mix thoroughly. Read the optical density at a wave length of 560 millimicrons in the spectrophotometer. For precise work, it is necessary to prepare chromatographed solvent blanks. The standard contains 50 micrograms of cholesterol in 3 milliliters of acetic acid, and the blank for this, is not chromatographed. The color develops rapidly and is stable for hours.

Difficulty in calibration was encountered in this procedure. The Bausch and Lomb Spectronic "20" was used, and the results of the calibration are recorded in Table III.

Analysis of the following chart reveals complications in this part of the procedure. It was noted that during the addition and mixing of the color developing reagent to the acetic acid solution of cholesterol a fair amount of heat was released. The maximum temperature reached was considered as a possible factor in the amount of color developed with any given standard. To study the effect of temperature on the color development, different cuvettes of the same standard acetic acid cholesterol solution were treated as follows: In number 1, the cuvette containing the 1 milliliter of the standard was heated to 90 degrees centigrade in a water bath before adding the color developing reagent; in number 2, the standard and the color developing reagent were mixed very rapidly; in number 3, the two solutions were mixed very slowly; in number 4, the cuvette was held under cold running water while the two liquids were mixed slowly. The results are indicated in Table IV. The standard used contained 36.72 micrograms of cholesterol per milliliter.

TABLE III. RESULTS OF CALIBRATING THE SILICIC ACID COLUMN CHROMATOGRAPHY METHOD OF CHOLESTEROL ANALYSIS

Micrograms of Cholesterol per Cuvette	Per cent Transmittance Obtained			
	Run 1	Run 2	Run 3	Run 4
4.59	86	87	98	99
9.18	97	98	91	98
13.77	89.5	95	96	90
18.36	82.5	83	83	83
22.95	93	86	80	81
27.54	84	91	84	88
32.13	77	82	85	77
36.72	83.5	74	73	81.5
86.85	55.5	65		
91.44	56	59.5		
96.03	49.5	53		
100.62	48.5	49		
105.21	51	53.5		
109.80	46.5	41		
114.39	42	43.5		

TABLE IV. PER CENT TRANSMITTANCE VALUES OBTAINED FROM MIXING, UNDER DIFFERENT CONDITIONS, A CHOLESTEROL STANDARD WITH THE COLOR DEVELOPING REAGENT.

Tube Number	Condition	Per cent Transmittance
1	Standard Heated to 90°C.	70.5
2	Mixed Rapidly	74
3	Mixed Slowly	83
4	Standard Cooled	95

Discussion and Further Studies

Examination of the results indicates that the rapidity with which the two fractions are mixed and the maximum temperature reached are important factors in the color development. Because it is difficult to control the amount of mixing when the color developing reagent is added to the acetic acid, this portion of the procedure was abandoned.

Duplication was easily obtained in the use of the analytical procedure by Sperry and Webb (32); therefore it seemed advisable to combine the silicic acid method of separating the two cholesterol fractions (33) and the Sperry-Webb color development method. In this combined method it was necessary to use a smaller dilution because a larger quantity of cholesterol is needed for the Sperry-Webb color development. Otherwise the chromatographic portion remained the same.

As a preliminary check on the accuracy of this procedure the per cent of cholesterol recovered from a known amount of cholesterol chromatographed was measured. To accomplish this an alcohol-acetone (1:1) solution of cholesterol was prepared containing 0.1706 milligrams of cholesterol per milliliter. The following volumes were placed in tubes, evaporated to dryness on the steam table, and chromatographed as above: 0.5 milliliters, 1.0 milliliters, and 1.5 milliliters. A blank prepared by passing an additional six milliliters of Skelly Solve B through the column at the beginning of the chromatographic procedure was chromatographed. The information obtained is indicated in Table V.

This procedure was repeated and similar results obtained. Nearly one hundred per cent of the cholesterol was recoverd which indicated that this method of analysis for free cholesterol was satisfactory. The value of this method of analysis for esterified cholesterol will be shown later.

TABLE V. CHOLESTEROL RECOVERY USING THE SILICIC ACID COLUMN AND THE COLOR DEVELOPMENT OF SPERRY-WEBB.

Milliliters of Standard Chromatographed	Results Obtained* Esterified	Free	Actual Cholesterol Concentration*
0.5	0	.085	.0854
1.0	0	.171	.1706
1.5	0	.257	.2569
0.0	0	0	0

* Concentration in milligrams chromatographed.

OPTIMUM HYDROGEN ION CONCENTRATION REQUIREMENTS FOR CHOLESTEROL ESTERASE

As stated before, the esterification of cholesterol with many acids has been studied; however, no study of cholesterol esterification with the essential fatty acids has been noted. In view of the importance of the polyunsaturated fatty acids and cholesterol in the condition of atherosclerosis, such a study would be interesting, although the results of such a study would not be conclusive as to the cause of the disease.

Swell and Treadwell (20) conducted studies on the optimum pH for esterification of cholesterol with a large number of fatty acids using a pancreatin extract as the enzyme source. In general, the pH values for the acids were at about the same level with little variation. However, a greater difference was noted between the optimum pH values for the esterification with stearic (pH of 5.5) and oleic (pH of 6.1) acids. It was of interest to see whether greater unsaturation of the fatty acids would bring about still higher optimum pH values.

Esterification of Cholesterol with Oleic Acid

Experimental

At varying pH values the esterification of cholesterol with oleic acid was determined in this laboratory using practically the same substrate and enzyme extract as Swell and Treadwell (20) did in their studies. The substrate was prepared as follows: A series of 0.154 molar phosphate buffers were prepared having pH values of 6.0, 6.2, 6.4,

6.6, 6.8, 7.0 and 7.2. One and one-half grams of cholesterol and 3.93 milliliters of oleic acid (the equivalent of which is three times the equivalent of cholesterol, were dissolved in 30 milliliters of ether. Ten milliliters of the appropriate buffer, 1 milliliter of 10 per cent sodium taurocholate, 1 milliliter of a 1:1000 aqueous solution of merthiolate, 500 milligrams of impalpable egg albumin, and 1 milliliter of the other solution were placed in a Potter-Elvehjem homogenizer and homogenized for two minutes. The material was then transferred to a beaker which was placed in a metabolic shaker (a Dubnoff Metabolic Shaker was used here) at 37 degrees centigrade for 1 hour to remove the ether. After the mixture had been shaken for 1 hour the pH was measured and adjusted with 0.1 normal sodium hydroxide to the values indicated in Table VI.

For the enzyme extract a 20 per cent suspension of Pancreatin (U.S.P.) was prepared in the homogenizer with a 1:1 dilution of glycerin and water. This was slowly mixed by continuously inverting the container for a period of about 30 minutes and was then centrifuged at 2500 revolutions per minute for 10 minutes.

It has previously been proved that this suspension of cholesterol is stable and remains stable during incubation with the enzyme (21). As a check on the substrate prepared in this laboratory, routine determinations of cholesterol were performed before and after incubation. To be certain that no esterification took place in the absence of the enzyme extract, the substrates having pH values of 6.6 and 6.8 were run with a blank; that is, only glycerin and water were added to duplicate quantities of substrate in place of the enzyme extract.

Five milliliter quantities of each substrate were pipetted into appropriate bottles. 0.5 milliliters of enzyme extract was added, the solution was mixed, and a 0.5 milliliter volume was removed and placed in a 25 milliliter volumetric flask for cholesterol analysis. The bottles were then capped and placed in the metabolic shaker, which had been previously adjusted to a temperature of 37 degrees centigrade, and were agitated at the rate of 120 oscillations per minute for 18 hours. About 15 milliliters of alcohol-acetone (1:1) were placed in the flasks with the 0.5 milliliter aliquot and heated to boiling on the steam table. The solution was then cooled, the flasks were filled to the 25 milliliter mark with alcohol-acetone mixture and contents were filtered. One milliliter of the filtrate was removed for evaporation and subsequent analysis with the silicic acid chromatography method.

At the end of the incubation period another 0.5 milliliter volume was removed, placed in a 25 milliliter volumetric flask, and treated as above. Here 2 milliliters instead of 1 milliliter of the filtrate were removed for chromatography because it was anticipated that the cholesterol would be divided into two fraction, requiring a larger quantity in each fraction for proper color development. The analyses of the filtrates gave the results indicated in Table VI.

TABLE VI. CHOLESTEROL ESTERIFICATION WITH OLEIC ACID

Sample Number	pH	Cholesterol Before Incubation (Free)*	Cholesterol After Incubation Ester-ified	Free	Total	Per cent Esterification
1	6.0	5.45	3.42	1.90	5.32	64.4
2	6.2	5.23	3.72	1.40	5.12	72.7
3	6.4	5.75	3.87	1.60	5.47	70.7
4	6.6	5.38	2.89	2.40	5.29	54.7
4B#	6.6	5.36	0.02	5.23	5.34	00.4
5	6.8	5.19	1.20	4.00	5.20	23.8
5B	6.8	5.23	0.00	3.95	3.95	00.0
6	7.0	5.30	3.20	2.15	5.35	59.9
7	7.2	5.38	0.30	5.05	5.25	3.51

*No value for esterification cholesterol was obtained.
#B Indicates that no enzyme was added.

Results and Discussion

Swell and Treadwell (20) performing similar experiments using the same substrate showed that the total cholesterol in suspension did not deviate throughout the incubation period. The results shown in Table VI indicate that with this method of analysis the total cholesterol (or free cholesterol) before incubation nearly equals the total cholesterol after incubation. This suggests that the method is satisfactory for analysis of free cholesterol. The values given as total cholesterol are the sum of the esterified and free cholesterol. It was previously shown that this method was satisfactory for analysis of free cholesterol. Therefore the analysis of esterified cholesterol must also be fairly accurate.

The results also indicate that the optimum pH for oleic acid in this study is between 6.0 and 6.4 which is consistent with the results of Swell and Treadwell (20). The results also show that very little if any esterification takes place in the absence of the pancreatic extract. Because of the great difference between the results obtained for the pH of 7.0 and the results of the two adjacent pH values and because this difference is not consistent with results obtained by others, the value obtained here for this pH may perhaps be disregarded. The value of 3.95 in number 5B (see Table VI) for free cholesterol after incubation is also not consistent and should very likely be disregarded.

Esterification of Cholesterol with Linoleic Acid

Experimental

For the esterification of cholesterol with linoleic acid a small quantity of high purity acid was used. This was obtained from the University of Minnesota and showed the following purity (35):

> "Prepared from highly purified methyl linoleate by saponification and distillation of the acids.
>
> Iodine value (Wijs) 181.0 (theoretical 181.03)
>
> Conjugated polyunsaturated constituents (from ultraviolet absorption data) expressed as percentage of C_{18} fatty acids.
>
> | | |
> |---|---|
> | Dienoic: | not more than 0.15% |
> | Trienoic: | not more than none |
> | Tetraenoic: | not more than none" |

The substrate was prepared for this analysis as it had been for the analysis with oleic acid; however smaller quantities were homogenized and no controls were used. 1.153 grams of the acid and 0.4610 grams of cholesterol were dissolved in ether and diluted to 7.2 milliliters. Six milliliters of the appropriate buffer, 0.6 milliliters of 10 per cent sodium taurocholate, 0.6 milliliters of 1:1000 merthiolate, 0.6 milliliters of the ether solution and 300 milligrams of egg albumin were homogenized. This mixture was placed in the metabolic shaker at 37 degrees centigrade for 1 hour and the pH adjusted to the values indicated in Table VII. The enzyme extract was prepared as before, and 0.4 milliliters of this was added to 5 milliliters of the mixture. After mixing, 0.6 milliliters was removed and placed in a 25 milliliter volumetric flask for extraction. A 2 milliliter aliquot was taken for chromatography. After incubation at 37 degrees centigrade in the

metabolic shaker for 18 hours with constant agitation of 120 cycles per minute, another 0.5 milliliter sample was taken from each bottle and placed in a 25 milliliter volumetric flask for extraction. Two milliliters of the filtrate was taken for evaporation and chromatography. Analysis of the 2 milliliter quantities for free and esterified cholesterol with the method using the silicic acid column separation and the sulfuric acid-acetic anhydride color developing reagent gave the results reported in Table VII.

TABLE VII. CHOLESTEROL ESTERIFICATION WITH LINOLEIC ACID.

Sample Number	pH	Cholesterol Before Incubation (Free)*	After Incubation			Per cent Esterification
			Esterified	Free	Total	
1	5.8	5.57	3.86	1.75	5.41	71.3
2	6.0	5.50	3.92	1.50	5.42	72.4
3	6.2	1.99	4.08	1.32	5.40	75.6
4	6.4	5.30	4.32	1.05	5.37	80.5
5	6.6	5.23	3.42	1.95	5.37	63.8
6	6.8	5.45	1.50	3.88	5.38	27.9
7	7.0	5.50	0.45	4.90	5.35	8.4
8	7.2	5.30	0.10	5.20	5.30	1.9
9	7.4	5.15	0.0	5.10	5.30	0.0
10	7.6	5.37	1.12	4.10	5.24	21.5

*No value for esterification cholesterol was obtained.
Values are expressed in milligrams per milliliter of original substrate.

Results and Discussion

In Table VII the value of 21.5 per cent esterification of cholesterol for the pH of 7.6 is not consistent with the curve; therefore, the value is probably in error and should perhaps be disregarded. The results have indicated an optimum pH between 6.2 and 6.6.

Esterification of Cholesterol with Linolenic Acid

Experimental

The study of esterification of cholesterol with linolenic acid was conducted in exactly the same way as the study of cholesterol esterification with linoleic acid except for the following differences: In the preparation of the substrate mixture 0.4601 grams of cholesterol and 1.183 grams of linolenic acid were dissolved in ether and diluted to 7.25 milliliters. As before, 0.6 milliliters of this solution was used in the preparation of the mixture. The other change consisted of using 3 milliliters of the filtrate resulting from cholesterol extraction of the samples taken after incubation for chromatography. Here, as with the esterification of cholesterol with linoleic acid, a high purity acid was used. This was also obtained from the University of Minnesota and showed the following purity (36):

> "Prepared from highly purified methyl linolenate by saponification and distillation of the acids.
>
> Iodine value (Wijs): 273.0 (theoretical value, 273.51)
>
> Conjugated polyunsaturated constituents (from ultraviolet absorption data) expressed as percentage of C_{18} fatty acids.
>
> | | |
> |---|---|
> | Dienoic: | not more than 0.20% |
> | Trienoic: | not more than trace |
> | Tetraenoic: | not more than none. |

TABLE VIII. CHOLESTEROL ESTERIFICATION WITH LINOLENIC ACID

Sample Number	pH	Cholesterol Before Incubation (Free)*	Cholesterol After Incubation			Per cent Esterification
			Esterified	Free	Total	
1	5.75	5.23	3.66	1.66	5.32	69.0
2	6.0	5.42	3.82	1.53	5.35	71.4
3	6.2	5.42	3.89	1.53	5.42	71.8
4	6.4	5.38	4.10	1.25	5.35	75.8
5	6.6	5.23	4.14	1.17	5.31	78.0
6	6.8	0.10	3.98	1.38	5.36	74.4
7	7.0	5.23	3.43	1.75	5.18	66.3
8	7.2	5.75	1.71	3.57	5.28	32.5
9	7.4	5.32	0.28	5.04	5.32	5.2
10	7.7	5.23	0.15	4.97	5.12	2.9

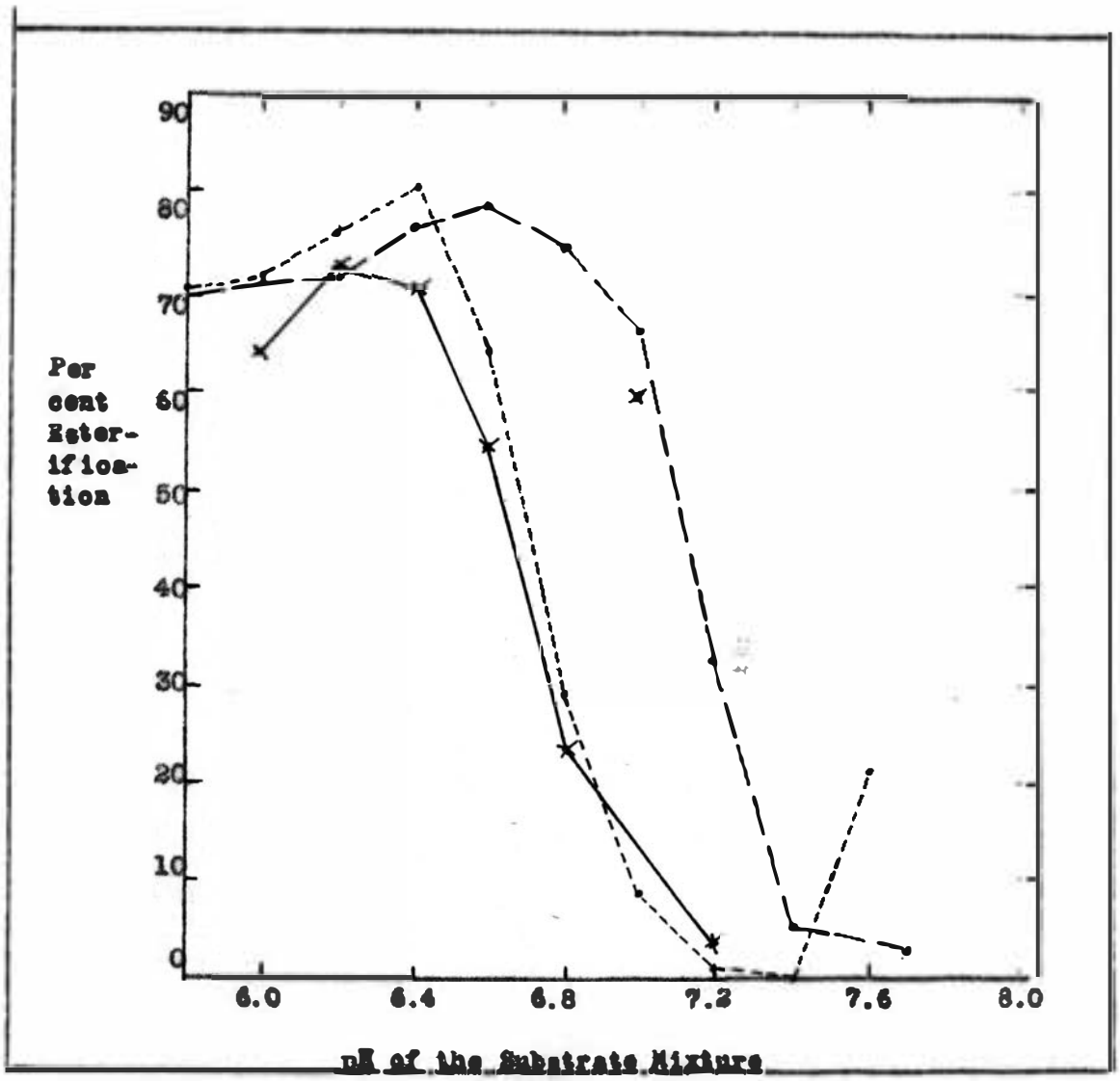
*No value for esterification cholesterol was obtained.
Values are expressed in milligrams per milliliter of original substrate.

The results obtained from the analysis appear in Table VIII.

Results and Discussion

In determination number 6 (see Table VIII) the value of 0.10 milligrams of cholesterol per milliliter of the substrate is probably an analytical error and should perhaps be disregarded. These results show an optimum pH between 6.4 and 6.8.

It was of interest to determine whether the optimum pH for the esterification of cholesterol would more closely approach the pH of blood and that of the intracellular fluid if the unsaturation of the fatty acid was increased. Graph 1 represents the results of the three esterification determinations.



Graph 1 Esterification of Cholesterol with Oleic, Linoleic and Linolenic Acids.

Linoleic Acid ----------
Linolenic Acid —— —— ——
Oleic Acid ————————

SUMMARY

In preparation for the study of cholesterol esterase difficulty was encountered in the method chosen for cholesterol analysis. Utilization of the Sperry-Webb method proved unsatisfactory in its entirety. However, the color development portion seemed satisfactory. Study of another method utilizing a silicic acid column for separation of the cholesterol fractions and subsequent color development with a ferric chloride-hydrochloric acid-sulfuric acid reagent also appeared unfavorable. In the latter method the color development portion presented the difficulty. A combination of the silicic acid column chromatographic method of separation of the fractions of cholesterol and the color development used in the Sperry-Webb method produced a sensitive, precise, workable method for quantitative analysis.

The optimum pH requirements for the enzyme cholesterol esterase were studied in relation to the esterification of cholesterol with oleic acid, linoleic acid, and linolenic acid.

Study of acids having the same carbon length but having greater unsaturation revealed higher pH requirements than previous studies had shown. The optimum pH for cholesterol esterification with oleic acid was between 6.0 and 6.4; with linoleic acid it was between 6.2 and 6.6; and with linolenic acid it was between 6.4 and 6.8. This shows that the higher the degree of unsaturation, at least up to 3 double bonds in 18 carbon fatty acids, the greater is the optimum pH of esterification.

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