DIETARY CHOLESTEROL ON FREE AND BOUND
CHOLESTEROL AND LIPID-PHOSPHORUS
CONTENT OF RABBIT AND RAT
AORTA, HEART MUSCLE,
PLASMA AND
ADRENAL

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
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By

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* * * * *

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I. INTRODUCTION

Degenerative arterial changes were present among the ancients. In 1911 Ruffer (1) examined Egyptian mummies dating from 1550 B.C., and found evidence of arterial calcification and atheromatous ulcers. The earliest written record pertaining to arteriosclerosis appears in writings of the anatomists of the sixteenth century (2). During the seventeenth and eighteenth centuries attention was focused mainly on observing the sclerotic and atheromatous state in various larger arteries of animals in general and man in particular, at autopsy. A review tracing the development of our knowledge of arteriosclerosis from the time of Vesalius to the nineteenth century has been written by Long (2). Early in the nineteenth century Lobstein (3) introduced the term arteriosclerosis, to designate the pathological condition characterized by thickening and hardening of the blood vessel wall, and in 1847 Vogel (3) demonstrated the presence of cholesterol in the atherosclerotic plaques of human arteries. This he accomplished by utilizing the fact that cholesterol crystals are birefringent when viewed under polarized light. In 1852 Johnson (2) called
attention to the thickening of the walls of the arterioles, and in 1903 Mönckeberg (3) described medial calcinosis which is confined primarily to the muscular arteries. There has thus emerged a triad of arteriopathies, each one primarily affecting a different portion of the arterial system. The term atherosclerosis, introduced by Marchand (3) in 1904 to designate the condition characterized by the presence of intimal lipid plaques which occur mainly in the aorta, and in the coronary and cerebral arteries, is regarded as the chief cause of mortalities due to cardiovascular derangements (3). The Committee on Nomenclature of the American Society for the Study of Arteriosclerosis has suggested (4) that the term arteriosclerosis be used in a generic sense, as originally introduced by Lobstein, to embrace among others, the three types of arterial pathology referred to above, i.e., intimal atherosclerosis, medial (Mönckeberg's) calcinosis, and arteriolar sclerosis.

The direct experimental approach to the atherosclerosis problem did not come into being in a precipitous manner. The feeding of pure cholesterol to rabbits antedates deliberate attempts to produce experimental atherosclerosis in this species by dietary manipulation. Thus, Pribram (5) in 1906, fed rabbits cholesterol in connection with his studies on intestinal absorption of sterols. The condition of the arteries in his experimental animals is not reported. Two years later, 1908, Ignatowski unintentionally produced atherosclerosis in the rabbit by feeding a diet of meat and
eggs as part of an investigation into the effects of different diets on renal function (6). Keen observation on the part of Anitschkw prevented what might have been, in the hands of a less discerning investigator, an overlooked occurrence of great significance. This worker held the opinion that the animal protein contained in the experimental diet of the herbivorous rabbit was the atherogenic stimulus. Soon after, Stuckey, (6) and Wesselkin, (6), demonstrated that only those experimental diets rich in cholesterol gave rise to atherosclerosis. Thus the stage was set for the crucial experiments of Anitschkw and Chalatow who, in 1913, succeeded in producing intimal atheroma in the rabbit aorta by feeding pure cholesterol (6). This was the first unequivocal demonstration of the relation between dietary cholesterol and experimental atherosclerosis. Exogenous cholesterol, now having been accused as an atherogenic stimulus in, at least, the rabbit, soon found its way into the diets of other animals. Up to the present, however, rabbits and chickens are the only experimental animals known to be susceptible to atherosclerosis when the sole experimental procedure is a cholesterol-supplemented diet. Cholesterol feeding alone has been found to be ineffective in producing atherosclerotic plaques in dogs, hamsters, monkeys, guinea pigs and rats unless such feedings are accompanied by endocrine and/or additional dietary imbalances (7, 3, 9, 10, 11). This situation has engendered
restraint, on the part of some workers, to accept the experimentally produced lesion in the cholesterolized rabbit as being analogous to the spontaneously occurring human lesion (12, 13). In addition, certain differences that exist between the site of occurrence and the morphology of the rabbit atherosclerotic plaque, as compared with that which occurs in the human, have been magnified and used as support for arguments which deny the essential similarity of the two processes. Anitschkow, in 1933, apparently anticipating these criticisms, remarked (6):

It of course goes without saying that the atherosclerotic process in experimental rabbits is not absolutely identical with human atherosclerosis in all its details. None of these differences is of an essential nature. Some of them are to be explained by structural differences between human and rabbit arteries, and others by different mechanical conditions. One can only be surprised that in spite of such differences the total picture of the atherosclerotic changes in experimental rabbits resembles that of human atherosclerosis in so many ways.

To this it should be added that species differences may provide a means of obtaining deeper insight into fundamental biological processes. Such differences may represent altered expressions of a basically similar physicochemical system in response to environmental changes. By examining such differences in the light of this concept, it is conceivable that the total potential of the physicochemical system to respond to environmental alteration will be more fully elucidated.
After Anitschkow and Chalatow had prepared the way for a direct experimental approach to the atherosclerosis problem there followed intensive efforts, mainly on the part of investigative pathologists, to exploit the experimentally produced lesion in terms of gaining knowledge concerning its human counterpart (12, 14, 15, 16, 17). Only within the past two decades or so, has concerted attention been directed to the more strictly biochemical aspects of the atherosclerosis problem. Once the emphasis had shifted from the morphological to the chemical approach to the problem, attention was naturally focused upon the cholesterol content of the atherosclerotic aorta, both in humans (18, 19) and in experimental animals, e.g., rabbits (20, 21, 22). In 1940, Weinhouse and Hirsch called attention to the possibility that lipids other than cholesterol might have an influence in the genesis of atherosclerosis (23). They noted that the various lipid constituents in the human atherosclerotic aorta were present in approximately the same proportion as that occurring in the blood (24). The view was put forth that atherosclerosis might represent a response to a derangement in the interrelations among the lipid moiety, and not necessarily to a quantitative alteration in one lipid constituent (25). This is in accord with the fact that human atherosclerosis is not confined to hypercholesteremic individuals (3). It remains a fact, however, that insofar as the experimental animal is concerned, a necessary condition for the production of atherosclerosis is the
elicitation of a sustained hypercholesteremia, which is usually induced by dietary cholesterol. This is not to say, however, that hypercholesteremia is a sufficient condition per se, for it has been shown that when cholesterol-fed rabbits are injected with synthetic detergents, viz., Tween-80 and Triton A-20, atherosclerosis is prevented or retarded (26). This occurs in spite of the fact that the hypercholesteremia in these animals is greater than in the non-injected cholesterol-fed animals which do exhibit atherosclerosis. In the former, however, the increase in the blood level of cholesterol is paralleled by an increase in the phospholipid level, so that the cholesterol/phospholipid ratio remains constant. It appears that the relationship between the levels of cholesterol and phospholipid may bear on the problem. There are observations on humans which tend to support this viewpoint (3). It is interesting to note that this apparent antagonism between these two substances is not confined to the pathological process under consideration. Thus, Theorell (27) discusses the antagonistic effects of cholesterol and phospholipid on hemolysis and sedimentation rates, while Affonskii and Magistris (27) report that cholesterol aids, but lecithin retards, the diffusion of acids and alkalies into gelatin and agar gels. The ability of lecithin to stabilize oil-in-water emulsions was shown many years ago (28), and, more recently, the effectiveness of phospholipids in stabilizing serum lipid emulsions has been demonstrated (29). In view of the
aforementioned facts concerning cholesterol and the phospholipids it would seem profitable to consider both in any study of the atherosclerosis problem.

For some time, in this laboratory, comparative studies have been in progress on the oxidative metabolism of aorta and heart muscle from cholesterol-fed rabbits and rats (30, 31, 32, 33). The animals used in these as well as in the present studies were treated experimentally for the most part as described in the next section, entitled, "Methods." The present investigation which is an extension of these metabolic studies, was undertaken for the following reasons: (1) to aid in the interpretation of data accruing from a study of the oxidative metabolism of aorta and heart muscle from rabbits and rats fed dietary supplements of cholesterol and cottonseed oil; (2) to compare the analytical data for free and esterified cholesterol and lipid-phosphorus content of adrenal, aorta, heart muscle and plasma from rabbits and rats fed cholesterol and cottonseed oil diets with those fed cholesterol without a carrying agent; (3) to present uniform experimental and analytical methods for the two foregoing studies, with regard to the strain and sex of the animals, diet, dietary supplements, and laboratory conditions for simultaneous analyses of free and esterified cholesterol and lipid-phosphorus; and (4) to obtain experimental data for the various tissue analyses from the rat, a species refractory to cholesterol per se as an atherogenic stimulus.
With regard to the first item, statistically significant changes in the oxidative metabolism of both aorta and heart muscle from the atherosclerotic rabbit as well as from the cholesterol-atherosclerotically refractory rat, when compared with their controls, have been given preliminary reports (30, 31, 32, 33). For this reason it is highly suggestive for the interpretation of these data to determine whether there are any concomitant changes in the free and esterified cholesterol and lipid-phosphorus content of these tissues. This is all the more pertinent when it is recalled that oxidative metabolic changes have been obtained for these same tissues from the rat which is, unlike the rabbit, refractory to cholesterol athero sclerosis. The question is posed for the future: Are the experimental changes noted in the oxidative metabolism, and in the analyses for the cholesterol and lipid-phosphorus content of the tissues under consideration to be correlated with atherogenesis, either actual or incipient?

Relative to the second item, the present study follows a previous one (33) treating of the same biochemical analyses on the same type of tissues from the same strain of animal, under relatively identical experimental conditions, but with diets supplemented with cholesterol in cottonseed oil, instead of with cholesterol in ether. During the course of these analyses, as well as during the course of the studies on the oxidative metabolism of aorta and heart muscle from these animals, concern was
felt lest the former data did not represent a true cholesterol effect. Thus the present experiments were undertaken with the cholesterol in ether solution -- the latter substance being subsequently evaporated and the cholesterol adsorbed to the food particles. Were the present results, from a standpoint of the biochemical analyses of cholesterol and lipid-phosphorus, different from the former data which is based on cholesterol-in-oil as the dietary supplement, then a clearer picture for the study of oxidative metabolism might be afforded by having recourse to a comparative study of tissue respiration from animals, both rabbits and rats, on the two types of cholesterol-supplemented diets.

Concerning the third item, free and esterified cholesterol have been determined for the following tissues of rabbits and chickens (all subsequent references relating to the latter species are underlined): adrenal (23, 34, 44), aorta (23, 35, 38, 44), heart muscle (44), plasma (38, 39, 40, 42, 43, 44), and liver (23, 38, 43, 44). Total cholesterol analyses alone have been undertaken for the following rabbit tissues: aorta (36, 37), plasma (41), and liver (36, 37). Simultaneous lipid-phosphorus and total cholesterol values have been presented for the following tissues of the rabbit and chicken: adrenal (23, 44), aorta (23, 38, 44), heart muscle (44), plasma (38, 39, 40, 41, 42, 43, 44), and liver (23, 38, 43, 44). Thus it
is seen that simultaneous analyses for free and esterified cholesterol and for lipid-phosphorus content of aorta, adrenal, heart muscle and plasma exist for only one species of experimental animal, viz., the cholesterolized chicken. For the cholesterol-fed rabbit, the only simultaneous analyses undertaken have been those for aorta and plasma (35). As for studies on the cholesterol-fed rat the reports are limited to analyses of total cholesterol alone (45), and total cholesterol with simultaneous lipid-phosphorus (41) of plasma.

With reference to the last item listed, it is of interest to determine the cholesterol and lipid-phosphorus content of aorta and heart muscle from cholesterol-atherosclerotic resistant rats, which, in spite of their refractoriness, exhibit changes in oxidative metabolism of aorta and heart muscle under the influence of a cholesterol-in-oil supplemented diet. In addition, the introduction of such biochemical studies on this species, parallel to those for the susceptible rabbit, would afford a firm basis for comparison, at the biochemical level, of the response from each of two species exhibiting such divergent behavior to the same dietary supplement.
METHODS

RABBITS. Male albino rabbits of the New Zealand strain, were obtained from a local animal breeder (Carroll Blue, Plain City, Ohio) at the end of weaning and at which time they weighed between 0.9 and 1.1 kilograms. They were divided into three experimental groups based upon addition of a supplement to the basic diet of Red Rose Rabbit Pellets (John W. Eshelman & Sons, Circleville, Ohio): A) normal - no supplement (control), D) 1% cholesterol (Merck U.S.P.) dissolved in 10% ether (Mallinckrodt, Analytical Reagent), E) 10% ether (control). For groups D and E the indicated supplements were thoroughly mixed with the pellets, and the ether was immediately evaporated. The animals were maintained under the usual laboratory conditions, at a temperature of 22 ± 2°C. All groups received tap water and their respective rations ad libitum for a period of 9 to 21 weeks unless otherwise stated, during which time each animal ingested an average of 100 grams of food per day. All animals came to autopsy within a weight range of from 2.7 to 3.0 kilograms.

From noon of the day preceding sacrifice (from 19 to 22 hrs. post-prandial) all rations were withheld, thus the animals were considered to be in the post-absorptive state. The tissues analyzed were obtained from a given rabbit in the following manner: after being weighed to the nearest gram the unanesthetized animal was secured to a standard operating board. Three - 15 ml. blood samples were obtained by cardiac
puncture with a non-sterile, heparin-rinsed (1:10,000), 20 ml. syringe fitted with a 1½ in. 20 gauge needle. After withdrawal of the last blood sample, the animal was sacrificed by aero-embolism induced by intra-cardiac injection of ca. 50 ml. of air. The blood samples were distributed into 6 plastic, conical centrifuge tubes, and the plasma was separated to constant volume by centrifugation for ½ hour at a relative centrifugal force of 2330 by an International Clinical Centrifuge. The heart, adrenals and aorta were rapidly removed and placed in separate moist-chambers. These solid tissues were cleared of extraneous fat and fascia. The heart chambers were exposed and cleared of blood and occasional blood clots, and excess body fluids were removed by blotting with Whatman No. 1 filter paper. The adrenals and heart were rapidly weighed to the nearest 0.1 mg., the former with a Roller-Smith torsion balance, and the latter with a Christian Becker Chainomatic balance.

Since tissue samples were destined for both cholesterol and lipid-phosphorus analyses the following plan was adopted to ensure random distribution of the tissues between the two procedures: the members of a given pair of adrenals were alternated in use for cholesterol and for lipid-phosphorus analyses so that the right and left adrenals from all animals of a group were distributed evenly between the two determinations. The aortas were cut longitudinally, one half for lipid-phosphorus analyses. From a statistical viewpoint
each dietary group contained between 9 and 14 animals. Three samples of each tissue (heart muscle, plasma, adrenal) were obtained from a given animal for each of the two analytical procedures. Where a scarcity of aorta precluded this, this tissue was taken alternately in duplicate and single samples (groups A & E).

**Cholesterol analyses**

The cholesterol content of all tissues studied was determined by the method of Schoenheimer and Sperry (46). This method makes use of the fact that digitonin, a glycoside, combines with free, but not esterified cholesterol, with the subsequent formation of insoluble cholesterol digitonide. The color reaction employed in this method is that of Liebermann and Burchard wherein cholesterol, in the presence of acetic anhydride and concentrated sulfuric acid, produces a green color complex, the intensity of which is proportional to the amount of cholesterol present.

The method of extraction of the tissues was, for all practical purposes, identical with that to be described for lipid-phosphorus, with the exception that the extractant for cholesterol was a 1:1 acetone-alcohol mixture. All tissue samples were run in duplicate, and colorimetric readings were taken with a Bausch and Lomb spectrophotocolorimeter ("Spectronic 20"), at a wave length setting of 625\(\text{nm}\).

**Lipid-phosphorus analyses**

Lipid-phosphorus was determined by the Youngburg and
Youngburg modification (47) of the Fiske and SubbaRow technique (46). The principle involved in this procedure is as follows: phosphorus is freed from its organic binding by sulfuric acid and is then oxidized to phosphate by hydrogen peroxide. The inorganic phosphate, in the presence of molybdate and para-amino napthol sulfonic acid, produces a blue-green color complex, the intensity of which is proportional to the amount of inorganic phosphorus present. Therefore this procedure is amenable to photometric measurement.

Rabbit tissues were extracted as follows. Three 2 ml. samples of plasma were each placed into 25 ml. micro-Folin tubes, each of which contained ca. 15 ml. of 3:1 alcohol-ether mixture. One longitudinal half of the aorta was divided into three samples of ca. 150 mg. each, except in those instances, previously noted, where only two samples were obtainable. Three heart muscle samples of ca. 150 mg. each were obtained in such a manner that the three samples corresponded to tissue from the left ventricular wall, septum and right ventricular wall, respectively. All samples from a given tissue were designated in order by alphabetic symbols, e.g., "A," "B," "C." The aorta and heart muscle samples were transferred to appropriately labeled porcelain mortars each of which contained 2 gm. of clean, sized, acid-washed, sharp sand. Enough extractant (3:1 alcohol-ether) was added to wet the sand and tissue, and each tissue was ground
(with a porcelain pestle) to the consistency of a smooth paste. The mortars and pestles were then placed on a sand-bath at a temperature of from 60 to 80°C. After drying, the content of each mortar was quantitatively transferred, by means of a policeman, to an appropriately labeled 25 ml. micro-Folin tube. As a final step in the transfer each mortar and pestle was rinsed twice with ca. 5 ml. portions of the extractant, and these rinsings were added to the appropriate micro-Folin tube. The contents of all tubes were boiled for 1 minute over a steam-bath, brought almost to volume with extractant, then stoppered and set aside for over-night extraction.

Adrenal tissue, because of its friability, does not require the grinding described above; instead, a sample was macerated on the wall of its micro-Folin tube by means of a glass stirring rod. After addition of the extractant, the macerated adrenal was scraped from the walls of the tube with the stirring rod, and the mixture boiled and extracted as described above.

RATS. Young male albino rats of the Wistar strain were purchased from the Harlan Small Animal Industries, Cumberland, Indiana when 25 to 35 gm. in weight. They were divided into three experimental groups comparable to those previously described for rabbits. These were treated essentially in the same manner as were the aforementioned rabbits with the following exceptions. The basic diet was ground Purina Dog
Chow Checkers (Ralston Purina Company, St. Louis, Missouri). Rats were maintained on the indicated diets for a period of 8 to 9 weeks, during which time each rat consumed an average of 20 gm. food per day. Because of the paucity of tissue involved, sub-groups of six rats each were employed per experimental run. Thus for the experimental purposes, six rats, were treated as equivalent to one rabbit. Nine or 10 sub-groups of rats were placed on each dietary regimen, and either 54 or 60 rats were employed per group analysis. All tissue samples were pooled as described below. The following tissues were taken: 5 ml. of blood were obtained by cardiac puncture, as previously described, from each of the six rats in a sub-group. The animal generally succumbed, or failing to do so it was sacrificed by cervical disjunction. After centrifugation of the pooled blood, in the manner already outlined, two 2 ml. samples of plasma were each distributed for cholesterol and for lipid-phosphorus analyses. The heart, adrenals and aorta were removed and cleaned as previously described. Both adrenal and heart weights were recorded for each animal according to the method described for the rabbit. The procedure employed for pooling the various tissues from a given rat sub-group is summarized in Table 1 (page 17). Column 1 of this table lists the three tissues under consideration; succeeding columns list, respectively, the portion of a given tissue, its origin within the sub-group, the alphabetical designation appended
TABLE I

Distribution of aortas, heart muscle and adrenals for cholesterol and lipid-phosphorus analyses for each sub-group of six rats.

<table>
<thead>
<tr>
<th>TISSUE AND NOTATION</th>
<th>PORTION</th>
<th>RAT NO.</th>
<th>SAMPLE DESIGNATION</th>
<th>ANALYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>thoracic abdominal</td>
<td>1, 2</td>
<td>&quot;A&quot;</td>
<td>cholesterol</td>
</tr>
<tr>
<td></td>
<td>thoracic abdominal</td>
<td>3, 4</td>
<td>&quot;A&quot;</td>
<td>lipid-phosphorus</td>
</tr>
<tr>
<td>Aorta (Ar)</td>
<td>abdominal thoracic</td>
<td>1, 2</td>
<td>&quot;A&quot;</td>
<td>cholesterol or lipid phosphorus, alternately with each sub-group</td>
</tr>
<tr>
<td></td>
<td>combined</td>
<td>5, 6</td>
<td>&quot;B&quot;</td>
<td>cholesterol or lipid phosphorus</td>
</tr>
<tr>
<td></td>
<td>apex base</td>
<td>1 (2)</td>
<td>&quot;A&quot;</td>
<td>cholesterol (lipid-phosphorus)</td>
</tr>
<tr>
<td></td>
<td>base</td>
<td>2 (1)</td>
<td>&quot;A&quot;</td>
<td>cholesterol (lipid-phosphorus)</td>
</tr>
<tr>
<td>Heart muscle (He)</td>
<td>apex base</td>
<td>3 (4)</td>
<td>&quot;B&quot;</td>
<td>cholesterol (lipid-phosphorus)</td>
</tr>
<tr>
<td></td>
<td>base</td>
<td>4 (3)</td>
<td>&quot;B&quot;</td>
<td>cholesterol (lipid-phosphorus)</td>
</tr>
<tr>
<td>Adrenal (Ad)</td>
<td>apex base</td>
<td>5 (6)</td>
<td>&quot;C&quot;</td>
<td>cholesterol (lipid-phosphorus)</td>
</tr>
<tr>
<td></td>
<td>base</td>
<td>6 (5)</td>
<td>&quot;C&quot;</td>
<td>cholesterol (lipid-phosphorus)</td>
</tr>
<tr>
<td></td>
<td>right</td>
<td>1, 2, 3</td>
<td>&quot;A&quot;</td>
<td>cholesterol</td>
</tr>
<tr>
<td></td>
<td>left</td>
<td>1, 2, 3</td>
<td>&quot;A&quot;</td>
<td>lipid-phosphorus</td>
</tr>
<tr>
<td></td>
<td>left</td>
<td>4, 5, 6</td>
<td>&quot;B&quot;</td>
<td>cholesterol</td>
</tr>
<tr>
<td></td>
<td>right</td>
<td>4, 5, 6</td>
<td>&quot;B&quot;</td>
<td>lipid-phosphorus</td>
</tr>
</tbody>
</table>
to the sample for purpose of future reference and, finally, the analysis performed upon the sample. The rat tissue samples were extracted for cholesterol and for lipid-phosphorus in the same manner as previously described for the rabbit tissue samples.

Following over-night extraction the contents of all tubes were brought to volume with extractant. The content of each tube was then filtered through Whatman No. 41 filter paper, and the filtrate collected in a 50 ml. erlenmeyer flask. To prevent loss of material due to absorption each piece of filter paper was wetted with extractant prior to filtration. To minimize evaporation, each funnel was covered with a watch-glass during filtration. In addition to the tissue samples, 25 ml. of extractant was filtered in the manner described above, and this filtrate constituted the "true blank" which subsequently was treated in all respects as a tissue sample. Two 5 ml. samples from each filtrate were placed into separate 50 ml. Pyrex culture tubes so that from this point on, all tissue samples and the true blank were run in duplicate. The culture tubes were placed over a steam-bath to completely evaporate the extractant. In order to free the phosphorus from its organic binding, 2.5 ml. of 5 N sulfuric acid were added to the residue remaining after evaporation, and the mixture was boiled until a few (3 to 5) fine charred particles appeared; immediately, 30% hydrogen peroxide was added, drop by drop, until the solution became water clear. The contents of each culture tube were
quantitatively transferred to 25 ml. micro-Folin tubes, and brought to volume with pyrex-glass distilled water.

Two sets of previously calibrated absorbency tubes were prepared for photometry. These were labeled to correspond to the duplicate tissue samples and true blanks and included, in each set, two additional tubes designated, "water blank" and "standard." Added to each of the former were 10 ml. distilled water, and to each of the latter 0.1 ml. standard phosphate solution (0.08 mg. PO₄/ml.) along with 7.9 ml. pyrex-glass distilled water and 1.5 ml. ammonium molybdate I ("standard molybdate"). To the true blank tube and to each of the tubes designated by a tissue sample were added 8 ml. of the solution from the correspondingly labeled micro-Folin tube, along with 1.5 ml. ammonium molybdate II ("tissue-molybdate"). At this point the electrical circuit of a spectrophotocolorimeter was turned on. The colorimeter was fitted with a red filter and was adjusted to a wave-length setting of 660 ml. Each of the absorbency tubes, with the exception of the water blank, received 0.5 ml. of 0.25% para-amino napthol sulfonic acid (PANS). Simultaneous with the addition of PANS to the first absorbency tube, an interval timer, calibrated in seconds, was turned on. The inside walls of all tubes receiving PANS were washed down by rotating the tubes about their longitudinal axes while in an almost horizontal position; following this each tube was "wind-milled" three times to insure thorough mixing of the
contents. The outside walls of all absorbency tubes were wiped clean with gauze. The colorimeter was set to indicate 100% transmittance with the water blank. At 300 seconds, which corresponds to the point of maximum color intensity, the standard and true blank tubes were read, in that order. The colorimeter was set to indicate 100% transmittance with the true blank, and then all tissue unknowns were read. The reading of all tubes was completed within 100 seconds. When the interval timer indicated 500 seconds the reading of all tubes was repeated, as described; this second reading served to check the stability of the color complex. The first and second readings were performed by different individuals. This procedure virtually eliminated the possibility of obtaining false readings due to incorrect alignment of the absorbency tubes into the colorimeter; it also served as a check against the possibility of erroneous readings of the percent transmittance scale of the colorimeter. Since all samples were run in duplicate, the procedure outlined above was repeated for the duplicate samples with the second set of absorbency tubes. The spread of disagreement between the values of duplicate samples was always within a 5% range of error. The arithmetic mean of the percent transmittance for a given pair of tissue samples was determined and this mean value was used to translate the mean percent transmittance value into the milligrams percent of phosphorus in the sample (See Table 3, page 25).
**Calibration curve**

In order to convert percent transmittance into mg. phosphorus (P) per unit weight (or volume) of tissue it was necessary to determine the percent transmittance of standard solutions prepared to contain various known concentrations of phosphorus. When these data are plotted on semi-logarithmic paper with percent transmittance of light as a function of mg. P per ml. of standard solution, a linear relationship is established (see Figure 1, page 24).

A standard phosphate solution was prepared as follows: 1.404 gm. \( \text{KH}_2\text{PO}_4 \) (Reagent) dried (at 110°C.) was placed into a 2-liter volumetric flask. To this was added 20 ml. 10 N \( \text{H}_2\text{SO}_4 \) (Reagent), and the solution was brought to volume with pyrex-distilled water. This standard solution contained 0.08 mg. P/ml.

Two sets of absorbency tubes were labeled according to the notation originally assigned to each tissue and as given in uncombined form in columns 1 of Table 1 and 2. In Table 2 is presented the indicated volumes of standard phosphorus solution (column 2), pyrex-glass distilled water (column 3) and ammonium molybdate I (column 4) which were added in serial order. The colorimeter was turned on at least 5 minutes previously, and simultaneous with the addition of the indicated volume of PANS (column 5) to the first tube, the interval timer was started. The procedure for reading the percent transmittance of all tubes was identical to
TABLE 2

Summary of notation and content of absorbency tubes used to prepare the phosphate standardization curve.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
<td>8.0</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td>0.1</td>
<td>7.9</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>7.8</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>7.7</td>
<td>1.5</td>
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<td>0.8</td>
<td>7.2</td>
<td>1.5</td>
<td>0.5</td>
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</tbody>
</table>
that described elsewhere for the unknown tissue samples. Since for each tube the mgm. P/ml. was known, then the percent transmittance corresponding to a given tube could be plotted as a function of the concentration of phosphorus in that tube. A straight line was the best fit to average the various points so obtained; this resulted in a calibration curve (Figure 1, page 24) from which the mg. P/ml. could be read along the abscissa from a given percent transmittance value on the ordinates. From this curve a conversion table was constructed (Table 3, page 25) which facilitated the reading of the mg. of P/ml. corresponding to a given percent transmittance value.
FIGURE I

Phosphate standardization curve

Percent transmittance vs. mg. P/ml.
TABLE 3

Conversion table: Readings in (\%T) on spectrophotocolorimeter in terms of milligrams (mg.) of phosphorus (P) per milliliter (ml.) of solution

<table>
<thead>
<tr>
<th>%T</th>
<th>mg. P/ml. x 10^4</th>
<th>%T</th>
<th>mg. P/ml. x 10^4</th>
</tr>
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<td>12.9</td>
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<td></td>
</tr>
</tbody>
</table>
CALCULATIONS

A. Lipid-phosphorus

1. Tissue samples:
   \[ \text{mg. P/100 mg. (wet) tissue} = a \frac{b \cdot c \cdot d}{e \cdot g} \]

2. Plasma samples:
   \[ \text{mg. P/100 ml. plasma} = a \frac{b \cdot c \cdot d}{e \cdot g} \]

where, \( a \) = percent transmittance in terms of mg.P/ml. (Table III).

\( b = 25 \text{ ml. dilution of tissue in alcohol-ether solution (dilution No. 1).} \)

\( c = 31.25 \text{ ml. dilution of tissue in distilled water (25 ml.), and 8 ml. aliquot sample of latter brought to 10 ml. with 1.5 ml. tissue molybdate plus 0.5 ml. PANS (dilution No. 2).} \)

\( d = 100 \text{ mg. tissue.} \)

\( e = 5 \text{ ml. aliquot samples of dilution No. 1 employed.} \)

\( f = \text{mg. (wet) tissue taken for extraction.} \)

\( g = 2 \text{ ml. plasma taken for extraction.} \)

B. Statistical formulas

1. Mean:
   \[ M_x = \frac{\sum N_x}{N} \]

2. Standard deviation about the mean:
   \[ \text{S.D.} = \sqrt{\frac{\sum (M_x - x)^2}{N-1}} \]

3. Standard error of the mean:
   \[ \text{S.E.} = \frac{\text{S.D.}}{\sqrt{N}} \]
4. "t" test ("Student's" method):

$$t = \frac{M_x_1 - M_x_2}{\sqrt{(S.E._1)^2 + (S.E._2)^2}}$$

where, $M_x$ = a given mean value.

$x$ = a given observation.

$N$ = total number of observations.

S.D. = standard deviation about mean.

S.E. = standard error of mean.
III RESULTS

All experimental data for the dietary groups studied are presented in Tables 4 to 9. Herein are presented, for a given tissue, the free and esterified cholesterol and lipid-phosphorus content, and the total cholesterol/lipid-phosphorus (C/P) ratio. Heart weight/body weight and adrenal weight/body weight ratios are included, as are the statistical analyses pertaining to appropriate group comparisons for the foregoing analyses.

The organization of Tables 4 and 7 is as follows: line 1 identifies the chemical analysis performed on the tissues listed in line 2. The first column denotes the dietary groups, and the second column lists the three calculated statistics. In the succeeding columns, under a given tissue, are presented the arithmetic mean, the standard deviation and the standard error of the mean, respectively. Immediately below these group data, under "statistical analyses," are presented the comparisons between the means of the two dietary groups indicated in column 1. These data are expressed in column 2 in terms of percent difference (%) and its probability value (P).

Tables 5 and 8 are arranged as follows: line 1 identifies the ratio calculated for each of the four tissues listed in line 2. The first column indicates the various dietary groups, and the second column the statistics calculated for each tissue. The remaining columns under a given tissue contain
the arithmetic mean, standard deviation and standard error of the mean, respectively. Below these data, under "statistical analyses," are found the comparisons of the mean values between two dietary groups in column 1, and expressed in column 2 as percent difference (%) and probability value (P).

Tables 6 and 9 are organized in the following manner: line 1 identifies the organ weight/body weight ratio under study. The first and second columns indicate the various dietary groups, and the three calculated statistics, respectively. Subsequent columns contain the arithmetic mean, standard deviation and standard error of the mean, respectively, for both of the organ weight/body weight ratios studied. Below these data, under "statistical analyses," are presented the indicated comparisons between the means of two dietary groups as noted in column 1, and expressed in column 2 in terms of percent difference (%) and its probability value (P). The following summary of all the foregoing data (Tables 4 to 9) is limited to a consideration of the statistically significant results.

A. RABBITS.

1. Cholesterol (Table 4): In comparing the cholesterol data of the ether controls with the normal controls, groups E-A, one finds that the concentration of esterified cholesterol is decreased by 89% (P<0.01) in the aortas, but is increased by 54% (P<0.02) in the adrenals. Comparison of the cholesterol-
in-ether group with its ether control, groups D-E, reveals that the concentration of free cholesterol in group D rabbits is increased by 533% (P<0.001) in the aortas, by 86% (P<0.001) in the heart muscles, by 2,02% (P<0.001) in the plasmas and by 451% (P<0.01) in the adrenals. Relative to the same comparison, the concentration of esterified cholesterol in group D rabbits is increased by 20,900% (P<0.001) in the aortas, by 2,600% (P<0.001) in the heart muscles and by 1,251% (P<0.001) in the plasmas.

2. Lipid-phosphorus (Table 4): A comparison of groups E-A and D-E indicates no significant difference in the lipid-phosphorus concentration of the tissues studied, except that in the latter comparison there is an increase of 633% (P<0.001) in the lipid-phosphorus concentration of the plasmas of group D rabbits.

3. Cholesterol/lipid-phosphorus (C/P) ratio (Table 5): The C/P ratio was determined for all tissues under study, and a summary of the mean values for these data is presented in Table 5. A comparison of control groups E-A shows no significant change in the C/P ratio of the tissues studied. However, a similar comparison of groups D-E reveals that the C/P ratio is increased by 572% (P<0.001) in the aortas, by 294% (P<0.001) in the heart muscles and by 192% (P<0.001) in the plasmas of group D rabbits.

4. Organ weight/body weight ratios (Table 6): The adrenal weight/body weight and heart weight/body weight ratios
were determined for all animals, and these data are summarized in Table 6. There are no statistically significant differences shown upon a comparison of the mean values for these ratios between groups E-A. However, a comparison of groups D-E reveals a 221% (P<0.001) increase in the adrenal weight/body weight ratio of group D animals compared with their group E controls.

B. RATS

1. Cholesterol (Table 7): In comparing the cholesterol data of the ether controls with the normal controls, one finds that the concentration of free cholesterol shows a 67% (P<0.001) decrease in the plasmas and a 38% (P<0.01) decrease in the adrenals, compared with their controls. Relative to the same comparison, the concentration of esterified cholesterol is decreased by 57% (P<0.02) in the aortas of group E rats. Comparison of the cholesterol-in-ether and its control, groups D-E, reveals a decrease of 100% (P<0.001) in the concentration of esterified cholesterol in the heart muscles, and an 18% (P<0.01) decrease in the concentration of free cholesterol in the plasmas of group D rats.

2. Lipid-phosphorus (Table 7): A comparison of groups E-A, indicates significant decreases in lipid-phosphorus concentration amounting to 50% (P<0.001) for the aortas, 23% (P<0.02) for the plasmas and 29% (P<0.01) for the adrenals. Comparison of groups D-E reveals no significant change in the concentration of lipid-phosphorus for the tissues studied.
3. **Cholesterol/lipid-phosphorus ratio (Table 3):** A comparison of groups E-A shows significant increases in the C/P ratio amounting to 89% (P<0.05) for the aortas, 47% (P<0.01) for the heart muscles, 46% (P<0.01) for the plasmas and 36% (P<0.05) for the adrenals. A comparison of groups D-E reveals no change in the C/P ratio of the tissues under study.

4. **Organ weight/body weight ratios (Table 9):** As is shown by the comparison between groups E-A, there is a 17% (P<0.05) increase in the adrenal weight/body weight ratio in group E rats. In the comparison between groups D-E there is revealed a 12% (P<0.05) decrease in the adrenal weight/body weight ratio for group D rats. There is no change in the heart weight/body weight ratio for either of the two comparisons.
A = control groups of animals.

D = experimental group of animals receiving a dietary supplement of 1% cholesterol in 10% ether, the latter being immediately evaporated.

E = control group of animals receiving a dietary supplement of 10% ether, which was immediately evaporated upon addition.

f = free cholesterol.

e = esterified cholesterol.

S.D. = standard deviation about the mean.

S.E. = standard error of the mean.

% = percent difference between a given pair of mean values for the dietary groups indicated.

P = probability value for the difference between a given pair of means for the dietary groups indicated.

mg. = mg. of cholesterol or lipid-phosphorus per 100 mg. (wet) tissue weight or per 100 ml. plasma.

C/P = ratio of total cholesterol concentration to lipid-phosphorus concentration.

< = less than.

> = greater than.

All values considered positive unless preceded by a minus (-) sign.
TABLE 4

Summary of mean values of free and esterified cholesterol and lipid-phosphorus concentration in the indicated rabbit tissues from animals fed a "normal", a "cholesterol-in-ether" or an "ether"-supplemented diet

<table>
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<th>Dietary groups</th>
<th>Adrenal</th>
<th>Aorta</th>
<th>Heart</th>
<th>Plasma</th>
<th>Adrenal</th>
<th>Aorta</th>
<th>Heart</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHOLESTEROL, mg.%</td>
<td></td>
<td></td>
<td></td>
<td>LIPID-PHOSPHORUS, mg.%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>f</td>
<td>e</td>
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<td>e</td>
<td>f</td>
<td>e</td>
</tr>
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<td>A</td>
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<td>6.65</td>
<td>0.21</td>
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<td>0.13</td>
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<td>13.92</td>
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<tr>
<td></td>
<td>S.D.±</td>
<td>0.25</td>
<td>1.72</td>
<td>0.10</td>
<td>0.08</td>
<td>0.02</td>
<td>0.02</td>
<td>14.22</td>
</tr>
<tr>
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<td>0.03</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
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<td>20.05</td>
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<tr>
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<td>S.D.±</td>
<td>0.25</td>
<td>3.19</td>
<td>0.08</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
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<tr>
<td></td>
<td>S.E.±</td>
<td>0.08</td>
<td>1.16</td>
<td>0.03</td>
<td>0.01</td>
<td>0.003</td>
<td>0.003</td>
<td>5.40</td>
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</table>

STATISTICAL ANALYSES

| E-A | % 29  | 54  | 14 | -9 | 8  | -6 | 6  | 53  | 13 | 0  | -12 | -2 |
|     | P >0.10 | <0.02 | >0.40 | <0.01 | >0.30 | >0.05 | >0.80 | >0.30 | >0.30 | 1.00 | >0.30 | >0.80 |
| D-E | % 451 | 16 | 533 | 20,900 | 86 | 2,600 | 2,002 | 1,251 | -23 | 67 | -14 | 633 |
|     | P <0.01 | >0.50 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
Summary of mean values of C/P ratios in the indicated rabbit tissues from animals fed a "normal", a "cholesterol-in-ether" or an "ether"-supplemented diet

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<th>Statistics</th>
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<th>C/P RATIO</th>
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<th>Heart</th>
<th>Plasma</th>
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</thead>
<tbody>
<tr>
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<td></td>
<td>Mean</td>
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<td>14.51</td>
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<td>0.13</td>
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<td>8.25</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>118.84</td>
<td>74.57</td>
<td>8.64</td>
<td>62.34</td>
</tr>
<tr>
<td>D</td>
<td>S.D.±</td>
<td>64.25</td>
<td>23.64</td>
<td>3.92</td>
<td>21.95</td>
<td></td>
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<tr>
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<td>S.E.±</td>
<td>22.72</td>
<td>1.18</td>
<td></td>
<td>7.32</td>
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<tr>
<td></td>
<td></td>
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<td>73.03</td>
<td>11.00</td>
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<td>21.35</td>
</tr>
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<td>E</td>
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<td>7.44</td>
<td>0.47</td>
<td>16.97</td>
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<tr>
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<td>10.27</td>
<td>0.16</td>
<td></td>
<td>5.66</td>
<td></td>
</tr>
</tbody>
</table>

STATISTICAL ANALYSES

| E-A       | % | P     | 44 | >0.10 | 31 | >0.30 | 18 | >0.10 | 47 | >0.20 |
| D-E       | % | P     | 63 | >0.05 | 572 | <0.001 | 294 | <0.001 | 192 | <0.001 |
Summary of mean values of adrenal weight/body weight and heart weight/body weight ratios in the indicated rabbit tissues from animals fed a "normal", a "cholesterol-in-ether" or an "ether"-supplemented diet

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Statistic</th>
<th>Adrenal weight/body weight</th>
<th>Heart weight/body weight</th>
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</thead>
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<td>1.93</td>
</tr>
<tr>
<td></td>
<td>S.D.±</td>
<td>3.85</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>S.E.±</td>
<td>1.03</td>
<td>0.07</td>
</tr>
<tr>
<td>D</td>
<td>Mean</td>
<td>23.49</td>
<td>2.19</td>
</tr>
<tr>
<td></td>
<td>S.D.±</td>
<td>9.79</td>
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<tr>
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<td>S.E.±</td>
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<td>0.16</td>
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<td>Mean</td>
<td>7.31</td>
<td>2.49</td>
</tr>
<tr>
<td></td>
<td>S.D.±</td>
<td>2.69</td>
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</tr>
<tr>
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<td>S.E.±</td>
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**STATISTICAL ANALYSES**

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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>E-A</td>
<td>%</td>
<td>-16</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>&gt;0.30</td>
<td>&gt;0.20</td>
</tr>
<tr>
<td>D-E</td>
<td>%</td>
<td>221</td>
<td>-12</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>&lt;0.001</td>
<td>&gt;0.50</td>
</tr>
</tbody>
</table>
### TABLE 7

Summary of mean values of free and esterified cholesterol and lipid-phosphorus concentration in the indicated rat tissues from animals fed a "normal", a "cholesterol-in-ether" or an "ether"-supplemented diet

<table>
<thead>
<tr>
<th>Dietary groups</th>
<th>Statis-</th>
<th>Adrenal</th>
<th>Aorta</th>
<th>Heart</th>
<th>Plasma</th>
<th>LIPID-PHOSPHORUS, mg. %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>f</td>
<td>e</td>
<td>f</td>
<td>e</td>
<td>f</td>
<td>e</td>
</tr>
<tr>
<td>A</td>
<td>Mean</td>
<td>0.53</td>
<td>2.68</td>
<td>0.22</td>
<td>0.07</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>S.D.‡</td>
<td>0.20</td>
<td>0.82</td>
<td>0.03</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>S.E.‡</td>
<td>0.06</td>
<td>0.26</td>
<td>0.01</td>
<td>0.01</td>
<td>0.004</td>
</tr>
<tr>
<td>B</td>
<td>Mean</td>
<td>0.39</td>
<td>3.04</td>
<td>0.26</td>
<td>0.02</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>S.D.‡</td>
<td>0.06</td>
<td>0.78</td>
<td>0.03</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>S.E.‡</td>
<td>0.02</td>
<td>0.27</td>
<td>0.01</td>
<td>0.01</td>
<td>0.002</td>
</tr>
<tr>
<td>C</td>
<td>Mean</td>
<td>0.33</td>
<td>2.49</td>
<td>0.24</td>
<td>0.03</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>S.D.‡</td>
<td>0.07</td>
<td>0.59</td>
<td>0.03</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>S.E.‡</td>
<td>0.02</td>
<td>0.20</td>
<td>0.01</td>
<td>0.01</td>
<td>0.002</td>
</tr>
</tbody>
</table>

| E-A | % | -38 | -7 | 9 | -57 | 0 | 0 | -67 | 8 | -29 | -50 | -25 | -23 |
|     | P | <0.01 | >0.50 | <0.10 | <0.02 | 1.0 | 1.0 | <0.001 | >0.30 | <0.01 | <0.001 | >0.05 | <0.02 |

| D-E | % | -9 | 24 | -4 | -33 | 0 | -100 | -18 | 0 | 17 | 0 | -17 | 12 |
|     | P | >0.30 | >0.05 | >0.40 | >0.40 | 1.0 | <0.001 | <0.01 | 1.0 | >0.10 | 1.0 | >0.10 | >0.30 |
### TABLE 5

Summary of mean values of C-P ratios in the indicated rat tissues from animals fed a "normal", a "cholesterol-in-ether" or an "ether"-supplemented diet

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Statistics</th>
<th>Adrenal</th>
<th>C/P RATIO</th>
<th>Aorta</th>
<th>Heart</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>18.60</td>
<td>8.15</td>
<td>1.81</td>
<td>17.19</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td>S.D. ±</td>
<td>4.99</td>
<td>2.68</td>
<td>0.30</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S.E. ±</td>
<td>1.58</td>
<td>0.84</td>
<td>0.09</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>25.25</td>
<td>14.75</td>
<td>2.70</td>
<td>20.93</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>S.D. ±</td>
<td>6.06</td>
<td>7.03</td>
<td>0.63</td>
<td>1.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S.E. ±</td>
<td>2.02</td>
<td>2.49</td>
<td>0.21</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>25.27</td>
<td>15.39</td>
<td>2.67</td>
<td>25.19</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td>S.D. ±</td>
<td>6.14</td>
<td>8.42</td>
<td>0.84</td>
<td>6.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S.E. ±</td>
<td>2.14</td>
<td>2.61</td>
<td>0.28</td>
<td>2.27</td>
</tr>
</tbody>
</table>

**STATISTICAL ANALYSES**

<table>
<thead>
<tr>
<th></th>
<th>%</th>
<th>P</th>
<th>%</th>
<th>P</th>
<th>%</th>
<th>P</th>
<th>%</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-A</td>
<td>36</td>
<td>&lt;0.05</td>
<td>89</td>
<td>&lt;0.05</td>
<td>47</td>
<td>&lt;0.01</td>
<td>46</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>D-E</td>
<td>0</td>
<td>1.0</td>
<td>-4</td>
<td>&gt;0.50</td>
<td>1</td>
<td>&gt;0.90</td>
<td>-17</td>
<td>&gt;0.10</td>
</tr>
</tbody>
</table>
### TABLE 9

Summary of mean values of adrenal weight/body weight and heart weight/body weight ratios in the indicated rat tissues from animals fed a "normal", a "cholesterol-in-ether" or an "ether"-supplemented diet

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Statistic</th>
<th>Adrenal weight/body weight</th>
<th>Heart weight/body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Mean</td>
<td>9.70</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>S.D.†</td>
<td>3.83</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>S.E.‡</td>
<td>0.70</td>
<td>0.21</td>
</tr>
<tr>
<td>D</td>
<td>Mean</td>
<td>10.00</td>
<td>3.30</td>
</tr>
<tr>
<td></td>
<td>S.D.†</td>
<td>1.86</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>S.E.‡</td>
<td>0.54</td>
<td>0.06</td>
</tr>
<tr>
<td>E</td>
<td>Mean</td>
<td>11.40</td>
<td>3.20</td>
</tr>
<tr>
<td></td>
<td>S.D.†</td>
<td>1.39</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>S.E.‡</td>
<td>0.40</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**STATISTICAL ANALYSES**

<table>
<thead>
<tr>
<th></th>
<th>‰</th>
<th>P</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>E-A</td>
<td>17</td>
<td>&lt;0.05</td>
<td></td>
<td>&gt;0.40</td>
</tr>
<tr>
<td>D-E</td>
<td>-12</td>
<td>&lt;0.05</td>
<td></td>
<td>&gt;0.20</td>
</tr>
</tbody>
</table>
Analyses for free and esterified cholesterol and for lipid-phosphorus content of aorta, heart muscle, plasma and adrenal, from cholesterol-fed rabbits and rats, show that there is a species difference with regard to the concentration of cholesterol and lipid-phosphorus in these tissues.

**CHOLESTEROL.**

**Plasma:** A comparison of the plasma from rabbits fed a diet supplemented with cholesterol-in-ether with that from ether controls (D-E groups) shows 2,002 (P<0.001) and 1,251 (P<0.001) percent increases in free and esterified cholesterol, respectively. These results are in essential agreement with those already reported for cholesterol-fed rabbits (35, 39, 40, 42, 43). The occurrence of relatively insignificant differences in the various plasma values, is not surprising when one considers the variation in species, sex, methods of feeding, and analytical procedures.

The similar comparison, (D-E group), but for rats reveals that the plasma concentration of free cholesterol is 18% (P<0.01) lower than in their ether controls, while there is no change in the concentration of esterified cholesterol. This results in an over-all decrease in the total cholesterol concentration in the plasma of cholesterol-fed rats. The explanation for this seemingly paradoxical situation may reside in the well-known tendency for ether to spontaneously
form highly reactive peroxides under favorable conditions (49). The fact that fatty acids are oxidized in the presence of hydrogen peroxide was demonstrated many years ago (50, 51, 52, 53, 54). The basic rat diet used in the present study contains 5% neutral fat (55). It is suggested that the possible oxidative destruction of the dietary fat, resulting from the formation of peroxides in the ether used as a cholesterol solvent, would result in a cholesterol-supplemented diet which acts as though it were relatively fat-free. In experiments designed to study the effect of fat on cholesterol absorption in the rat, Cook (56) found that when cholesterol was fed with a relatively fat-free diet the sterol could be recovered quantitatively from the feces. In the present study, the absence of any significant absorption of cholesterol would account for the failure to obtain a rise in the level of cholesterol in the plasma of cholesterol-fed rats. The fact that the plasma cholesterol level in such rats is actually below that of their ether controls may possibly be the result of a decreased reabsorption of endogenous cholesterol under the prevailing dietary conditions. It has long been recognized that the presence of bile in the intestine is a necessary concomitant of lipid absorption. It was demonstrated many years ago that pancreatic lipase is activated by bile (57). The emulsifying properties of bile also contribute to their role in lipid absorption. Finally, the production of water-soluble coordination compounds of fatty acids with bile salts
is regarded by Deuel (58) as the most important action of the bile salts in facilitating the absorption of lipids from the intestine. The efficiency of lipid absorption apparently depends upon an optimum ratio between fatty acid and bile salt molecules; the smaller the ratio the more efficient the absorption (59). It has been reported that cholesterol, in addition to fatty acids, forms coordination compounds with bile salts (60). If it be assumed that efficient cholesterol absorption, like that of fatty acids, is dependent upon a small cholesterol to bile salt ratio, then one would expect that in the presence of a large excess of cholesterol the efficiency of absorption of the sterol would be less than if it were not present in excessive amount. This would account for the decreased level of plasma cholesterol in those rats fed the sterol dissolved in ether compared with the ether controls; in the former group, the presence of excess dietary cholesterol could conceivably raise the cholesterol to bile salt ratio to such extent as to markedly curtail not only the absorption of dietary cholesterol, but also the reabsorption of endogenous cholesterol. One may thus envision a loss, via excretion, of at least some endogenous biliary cholesterol which, had the usual dietary fat been present, would otherwise have been reabsorbed. It is pertinent to note at this point that the amount of cholesterol in rat bile has been reported as 12.7 mg.% (61). It has been demonstrated that there is a considerable amount of cholesterol synthesized by the mucosa
of the small intestine and colon of the rat (62). This second source of endogenous cholesterol, like the sterol present in bile, would suffer excretion and so contribute to the lowering of the plasma cholesterol level. This concept of the mechanism of the lowering of the plasma cholesterol level in rats fed cholesterol dissolved in ether would be supported experimentally could it be demonstrated that the plasma cholesterol level in the ether control animals, when compared to the normal controls, is markedly depressed. That this is, in fact, the case is shown by the comparison between groups E-A. The plasma concentration of free cholesterol is 67% (P<0.001) lower in the ether control animals when these are compared to the normal controls.

The fact that the absorption of cholesterol was apparently not impaired when cholesterol-in-ether was given to the rabbits confirms previous studies on this species (23, 42). However, the two latter studies were reported without the employment of ether controls. Furthermore, there are no reports in the literature concerning rats fed cholesterol-in-ether. However, a lowering of plasma cholesterol has been observed in normal rats maintained on a fat-free diet (63) as well as in rats whose sole dietary supplement was cholesterol (64). The present study demonstrates a striking species difference, between the rabbit and the rat, in the absorption of cholesterol. Rabbits, unlike rats, have been shown to absorb cholesterol on a low fat diet (65). That the
greater facility of the rabbit, when compared to the rat, to absorb cholesterol is not solely dependent upon the presence of dietary fat is shown by the fact that, in the presence of a given amount of dietary fat, rats absorb 46 to 50% and rabbits 32%, of dietary cholesterol (66). The elucidation of the mechanism involved in this species-determined difference in cholesterol absorption awaits future study.

Aorta: A comparison of cholesterol-in-ether with ether fed rabbits (D-E groups) reveals 533% (P<0.001) and 20,900% (P<0.001) increases in free and esterified cholesterol, respectively. Not only is the percent increase in esterified cholesterol greater than that for the free sterol, but the absolute amount of the former exceeds that of the latter, findings which are in agreement with the data of Weinhouse and Hirsch (23) and Dury (38) on this species. A comparison of rabbits fed ether with their normal controls (groups E-A) shows a decrease of 89% (P<0.01) in the concentration of esterified cholesterol in the aortas of the ether control animals as compared to the normal controls. It is unfortunate that previous investigators (23, 42), and, incidently, the "editors" did not deem it necessary to use or require ether controls. The present work suggests a possible biochemical derangement in at least some rabbit tissues resulting from the ingestion of a diet pretreated with ether and as employed in these and other experiments (23, 42). The factor, or factors, responsible for this
experimental anomaly can only be ascertained by future
investigation, but it appears not amiss, at this point, to
mention an observation which may be pertinent to the matter.
The tendency for ether to form peroxides has been referred
to previously, and in this connection it is of interest to
note the finding, by Altschul (10), that pretreatment of
dietary cholesterol with hydrogen peroxide resulted in a
marked decrease in the severity of rabbit tissue lipidosis.*
There was some involvement of the coronary arteries, but the
usual aortic plaques were not observed. The cursory obser­
vation that hydrogen peroxide itself, or some product of its
interaction with a dietary constituent, exerts an inhibitory
effect on the usual response of the rabbit aorta to dietary
cholesterol may have bearing on the present observation, viz.,
that rabbits maintained on a diet pretreated with ether exhibit
a lowered concentration of cholesterol in the aorta.

For similar studies on rats, the only significant change
observed is a 57% (P<0.02) decrease in esterified cholesterol
in the aortas of the ether controls, when these are compared
with normal controls (groups E-A). It is of interest that
this finding parallels exactly that for the rabbit aorta for
the same dietary comparison, i.e., for both species the ether
controls show similar and significant decreases in the content
of esterified cholesterol in the aorta, when compared with
their normal controls. Thus it appears that two species, the

*With one exception, which is discussed later in this
section, under "Adrenal."
rabbit and rat, antithetic though their responses be to a diet atherogenic to the former, show strikingly similar changes in the esterified cholesterol content of the aorta when under the influence of a diet pretreated with ether. It is of additional interest to note the recent demonstration that there is an increased rate of respiration in rat thoracic aorta from animals maintained on a cholesterol-supplemented diet (75), a finding, which is in keeping with a previous observation in this laboratory (30), that the aorta of the rabbit on such a diet also exhibits an increased rate of metabolism. These basic similarities in the response of a tissue, viz., the aorta, which is the focal point of the atherosclerotic condition, become the more intriguing when viewed in the light of the propensity of the rabbit aorta, and the resistance of the rat aorta, to succumb to the atherosclerotic lesion.

The aortas of cholesterol-fed rabbits in the present study, unlike the rats, exhibited, without exception, extensive involvement with atherosclerotic plaques. These discrete, macroscopically visible lesions occurred with greater severity in the thoracic portion of the aorta, but considerable involvement of the abdominal aorta was not an infrequent observation. On the other hand, careful examination of the aortas from rabbits not receiving dietary cholesterol revealed no evidence of any gross pathological change. The quantity of lipid material that accumulates
in the atherosclerotic lesions is so large as to preclude, in all likelihood, its origin from the breakdown of intimal tissue. Hirsch and Weinhouse (25) demonstrated, in the human, that both the normal intima and the early atherosclerotic plaque have essentially the same lipid distribution that obtains in the plasma. This observation gave great support to the "infiltration theory" of atherogenesis according to which the lipid constituents of the atherosclerotic lesion have their origin in plasma lipids which have permeated the endothelial lining of the artery. Recent work with the rabbit lends support to this concept (67). The mechanism involved in the localization of the atherosclerotic lesion is a problem which remains unanswered however, since the discrete distribution of the plaques must be an expression of local differences in the arterial wall, and the composition of the plasma that bathes the arterial endothelium is presumably everywhere the same. Thus although the amount and/or physicochemical state of the plasma lipids may determine whether or not the atherosclerotic lesion will occur, such considerations are apparently not involved in determining the site at which the lesion develops. Preoccupation with the level and/or state of the plasma lipids, to the exclusion of considerations of local metabolic factors in the arterial wall, is, therefore, not justified by the facts. The arteries of humans (68), rabbits (62), chickens (62) and calves (69) have been shown to be active in biochemical synthesis; such
facts compel one to look upon the arterial wall as playing more than merely a passive role in the genesis of the atherosclerotic lesion. In fact, differences in arterial biochemical activity have been suggested as a contributing factor, at least, in species-determined differences in susceptibility to atherosclerosis. Thus Zemplenyi et al. (70) have demonstrated differences in enzymatic activity, as measured by lipolysis of human lipemic serum, in the aortas of rats, guinea pigs and rabbits. Not only is there a species-determined difference in enzyme activity, but the locale of the enzyme system within the layers of the arterial wall varies from one species to another. The activity and the location of the lipolytic enzyme system are correlated with the degree of susceptibility of a given species to atherosclerosis. Even within a given species, viz., the chicken, different arteries of the same animal behave independently of each other. Thus, estrogen prophylactically inhibits coronary atherogenesis in cholesterol-fed chickens, whereas it is completely without effect on aortic atherogenesis (3). Recent observations on the rabbit suggest that in this species also, the coronaries and the aorta do not parallel each other in their atherogenic behavior (71). Finally, the demonstration, in the rat, that the thoracic and abdominal aorta have different rates of respiration (72) has obvious implications when it is recalled that the aorta of the human and the rabbit show a predilection, albeit
reciprocal, as to the site of development of the atherosclerotic lesions.

The diverse behavior of arterial tissue in different species, and, indeed, in a given animal, forcibly emphasizes the importance of local tissue factors as determinants of major import in the atherosclerotic process.

**Heart:** A comparison of rabbit groups D-E shows increases of 86% (P<0.001) and 2,600% (P<0.001) in the concentrations of free and esterified cholesterol, respectively, in the heart muscle of cholesterol-fed rabbits. Although no reports exist in the literature on the cholesterol content of heart muscle from cholesterol-fed rabbits, the present results are in agreement with those of Stamler, et al. (44) who determined the cholesterol content of heart muscle from chickens receiving dietary cholesterol. The coronary arteries of both the rabbit (10) and the chicken (3) are susceptible to atherosclerotic plaques under the influence of dietary cholesterol. This situation, in itself, could account for some of the increase in the cholesterol content of heart muscle from rabbits and chickens receiving dietary cholesterol. The fact that heart muscle fibers of cholesterol-fed rabbits become laden with lipids has been demonstrated by Altschul (10). This lipid infiltration of rabbit cardiac muscle may be a reflection of the possible inability of this particular tissue to oxidize lipids. It has been shown, for example, that there is a decreased oxygen consumption in cardiac
muscle from rabbits receiving a dietary supplement of cottonseed oil (73). Of course, the possibility exists that extensive lipid infiltration may result in less than optimal conditions in such a manner that any enzymatic activity that is characteristic of normal cardiac muscle may suffer curtailment in the face of excessive lipidosis.

The D-E group comparison for rats reveals a decrease of 100% (P<0.001) in the concentration of esterified cholesterol in the heart muscle of cholesterol-fed compared with the ether controls. The literature is without reports on the cholesterol content of cardiac muscle from the cholesterol-fed rat. In experiments performed in this laboratory (73) pertaining to the oxidative metabolism of heart muscle from rats fed a diet supplemented with cholesterol and oil, it was suggested that, in this species, the dietary oil functions as an esterifying agent with respect to the cholesterol molecule. Since the group D rats have a plasma cholesterol level which is below that of the group E animals, and, furthermore, since both groups of animals are presumably on a diet which is relatively fat-free, it is to be expected that the content of esterified cholesterol should be lower in the heart muscle from group D rats compared with that in the animals of group E.

**Adrenal:** In cholesterol-fed rabbits the concentration of free cholesterol is increased by 451% (P<0.01) over that in the ether controls (groups D-E). There is no change, in
the concentration of esterified cholesterol in the adrenals of group D animals. Since, in the adrenals of the normal rabbit, the esterified sterol is present in greater concentration than is the free form, the absolute amount of esterified cholesterol exceeds that of the free cholesterol in the cholesterol-fed rabbit. These findings are in accord with those of Weinhouse and Hirsch (23) and Kay and Whitehead (34). The cholesterol content of the adrenal cortex is large compared with that of other organs (55). The normally high content of cholesterol is presumably related to the synthesis of the adrenal cortical hormones, since it has been demonstrated (74) that cholesterol is transformed, by adrenal cortical tissue, into the hormones released by this gland. It is reasonable to expect, therefore, that a hypercholesteremia should be accompanied by an increase in the concentration of cholesterol in adrenal tissue. The E-A comparison for rabbits reveals a 54% (P<0.02) increase in the concentration of esterified cholesterol in the adrenals of the ether controls compared with normal controls. Previous reference has been made to Altschul's (10) treatment of cholesterol with hydrogen peroxide prior to incorporating the sterol into the diet of rabbits. It was noted that rabbits fed such pretreated cholesterol developed, in general, a lipidosis which was less intense than that obtained in rabbits receiving untreated cholesterol. The only organ not so spared was the adrenal cortex. According to Altschul (10):
The suprarenal cortex was the organ with most distinct parenchymatous changes, showing foamy transformation of cortical cells and in one rabbit large necrotic foci and cholesterol crystals.

The fact that hydrogen peroxide, or one or more of its interaction products, fails selectively to "protect" the adrenal gland in the rabbit may be correlated with the present finding that rabbits whose diet has been pretreated with ether exhibit a greater than normal content of cholesterol in the adrenal gland. Such cursory observations, while suggestive, nevertheless require the firm foundation of carefully controlled experiments to warrant anything more than speculation concerning the mechanism involved in these interesting findings.

For rats, the only significant change is revealed in the comparison between groups E-A. The adrenals of the ether control animals show a 35% (P<0.01) decrease in the content of free cholesterol compared with the adrenals of the normal controls. This finding is in contrast to the increase in cholesterol content of rabbit adrenals when the corresponding dietary groups are compared. Thus there appears to be a species-determined difference in the response of adrenal tissue from rabbits and rats maintained on a diet pretreated with ether. As pointed out earlier, these data admit of no such interpretation at present, but must await future study for their clarification.
LIPID-PHOSPHORUS.

In the interest of clarity and conciseness of presentation it was felt desirable to depart from the format used in the preceding discussion treating of the changes in cholesterol content of the various rabbit and rat tissues. The several data pertaining to the changes in lipid-phosphorus content of the tissues under study are of such nature that they are best discussed as a whole, but under separate headings, for the rabbit and the rat.

Rabbits: The only finding with respect to a significant change in the lipid-phosphorus content of rabbit tissue is an increase of 633% (P<0.001) in the plasma of the cholesterol-in-ether fed rabbits when these are compared with the ether controls (groups D-E). This finding is in essential agreement with previous reports on the cholesterol-fed rabbit irrespective of whether the dietary sterol was fed dry (38, 40), or dissolved in Crisco (43), cottonseed oil (39, 41) or ether (42). In the normal rabbit it has been found that not only the liver, but extrahepatic tissues as well, are the sources of plasma phospholipids (76). Under the influence of dietary cholesterol, however, the synthesis of phospholipid by the liver and aorta is greatly accelerated compared to other tissues, and the increase in the plasma phospholipids is due almost exclusively to donation by the liver (77).

The phospholipid content of the aorta of the cholesterol-in-ether fed rabbits deserves mention at this point. A
comparison of this group with their ether controls (group D-E) reveals a 67% increase in phospholipid content of the aorta. Although this increase just fails to show statistical significance (P>0.05), the concentration of phospholipid in the aorta of the cholesterol-fed rabbits in the present study is in excellent agreement with previous reports (23, 38) treating of the same analysis. It is tempting to conclude, a priori, that the source of the increased aortic phospholipids is the plasma circulating within the lumen of the artery. There is evidence, however, that points to the artery itself as contributing significantly to the contained phospholipids (77, 78).

Thus it appears that the two rabbit tissues, viz., liver and aorta, which are heavily infiltrated with lipid in the cholesterolized animal, each exhibit an increase in phospholipid synthesis. For the teleologist this situation requires no further comment since the phospholipids are known to be extremely effective as lipid emulsifiers (28, 29), and their presence in tissues heavily infiltrated with cholesterol would provide the obvious advantage of solubilization and dispersion. Reference has already been made to the finding that the aorta of the rabbit (30) and rat (75), under the influence of a cholesterol-supplemented diet, show an increase in oxygen consumption. It is possible that these tissues react to lipid infiltration by an increase in their rate of oxidation of the invading lipid. Phospholipids appear to participate in the oxidation of lipids (79), and
may so participate by virtue of their choline content, since
the latter substance is known to promote the oxidation of
fatty acids in liver (50) and in extrahepatic tissues (51). The view that increased phospholipid synthesis is attendent upon an accelerated rate of lipid oxidation would be strengthened upon the demonstration that the choline-containing phospholipids, viz., lecithin and sphingomyelin, are synthesized to a greater extent than is cephalin, a non-choline-containing phospholipid. It has been demonstrated, in the rabbit, that cholesterol-induced alterations in aortic phospholipid synthesis occur primarily in the lecithin and sphingomyelin fractions (52). Furthermore, these two choline-containing phospholipids were found to accumulate in the human atheromatous aorta (53).

The foregoing considerations should not be construed as an attempt to discredit the "infiltration" concept of atherogenesis. Data gathered from experiments with the rabbit (67) leave little doubt about the soundness of the proposal in, at least, this species. One may speculate that under normal conditions, with respect to the concentration and/or the physical state of the plasma lipids, and also with respect to the metabolic and/or physical integrity of the arterial wall, the lipids present in the plasma filtrate which have found their way into the arterial wall are removed, by way of the vasa vasorum and lymphatics of the vessel wall, at such a rate as to prevent their
accumulation. In the face of an alteration in the plasma lipids or in the arterial wall, or both, the rate of removal of infiltrated lipids would not keep pace with their entry and these would remain in the arterial wall to result in mischief therein.

Rats: The only significant changes in the concentration of lipid-phosphorus for rat tissues are those revealed by a comparison of groups E-A. Thus, in the ether control animals the lipid-phosphorus concentrations of plasma, aorta and adrenal are decreased by percentages of 23 (P<0.02), 50 (P<0.001) and 29 (P<0.01), respectively, compared with the normal controls. The possibility has been put forth, earlier in the present work, that rats whose food has been pretreated with ether have, as a result, been maintained on a relatively fat-free diet. It has been shown (34) that in the fasting rat the liver supplies the major amount of plasma phospholipids, but after feeding, particularly fats, the intestine contributes an appreciable amount of phospholipid to the plasma. This observation may serve to explain the decreased level of plasma phospholipid in the ether control animals compared to that in the normal control animals, inasmuch as the diet of the latter contained 5% fat (55). A second observation which is of interest in this connection is that of Artom (35) who showed that tocopherol accelerates the oxidation of fatty acids in rat liver. Tocopherol is one of the fat-soluble vitamins, and its
activity is destroyed by oxidation (86). In the light of
the assumption that the group E rats consumed a relatively
fat-free diet as a result of the possible oxidative
destruction of dietary lipids, the latter conceivably due
to peroxide formation in the ether employed as a control
measure, one may reasonably surmise that these group E rats
were subjected to a diet deficient in tocopherol. Since
the livers of tocopherol-deficient rats exhibit a depressed
rate of fatty acid oxidation (85) and, further, since lipid
oxidation is attended by an increased rate of phospholipid
synthesis (79), then the livers of tocopherol-deficient
rats would be expected to exhibit a decrease in their phos­
pholipid content. Since the liver supplies the major
fraction of the plasma phospholipids (84), a decline in liver
phospholipid would be reflected by a decrease in plasma
phospholipid. It is known that extrahepatic tissues oxidize
fatty acids (87), and it is to be expected, therefore, that
a depression of fatty acid oxidation in the aorta and in the
adrenal should be associated with a decrease in the content
of phospholipid in both these tissues.

The increased concentration of plasma phospholipids
which invariably obtains in the cholesterol-fed rabbit (38,
39, 40, 41, 42, 43) has been observed to occur also in the
cholesterol-fed chicken (44) and rat (41). In the present
study, the cholesterol-fed rabbits exhibit the expected
phospholipemia whereas the rats fail to do so, and a possible
explanation for the latter finding has been presented. The phospholipemia associated with cholesterol feeding has been considered as an expression of a compensatory mechanism which serves to solubilize the cholesterol molecule and so minimize its deposition in the arterial wall.

In the present work an attempt has been made to account for the increased level of tissue phospholipid in the cholesterol-fed rabbit as resulting from, and associated with, an increase in the rate of lipid oxidation, the latter representing a response by tissues having been infiltrated with lipid. The data on the cholesterol-fed rat, negative with respect to increases in tissue phospholipids, have been analyzed in the light of pertinent literature, and have been shown to be consistent with such a concept.

**CHOLESSTEROL/LIPID-PHOSPHORUS RATIOS.**

The C/P ratio was determined for all tissues studied, in both the rabbit and the rat. A consideration of the C/P ratio, following, as it does, in the wake of the foregoing discussion of the changes in cholesterol and lipid-phosphorus content of rabbit and rat tissues, may appear superfluous. However, it was felt desirable, for the sake of completeness, to present such data.

**Rabbits:** A comparison of groups D–E shows significant percent increases in the C/P ratios for aorta -- 572 (P<0.001), heart -- 294 (P<0.001) and plasma -- 192 (P<0.001). For the aorta and heart tissues of group D animals there were no
significant changes in the concentrations of lipid-phosphorus, hence the increase in the C/P ratio must be ascribed solely to the increase in cholesterol content which occurred in both these tissues. In the plasma of group D animals, however, there was a significant increase in the concentration of lipid-phosphorus so that in this case the elevation of the C/P ratio is a reflection of the fact that the rise in lipid-phosphorus concentration was not of sufficient magnitude to keep pace with the still greater increase in cholesterol content of the plasma of cholesterol-fed rabbits.

Rats: The comparison of groups E-A reveals significant increases in the C/P ratios of all tissues studied. Thus for aorta, heart muscle, plasma and adrenal the percent increases are, respectively, 89 (P<0.05), 47 (P<0.01), 46 (P<0.01) and 36 (P<0.05). For aorta, plasma and adrenal there are decreases in both the cholesterol and lipid-phosphorus content, but the decrease in the latter is greater in magnitude than that of the former, hence a consequential elevation of the C/P ratio in these tissues. For heart muscle there is a 25% decrease in lipid-phosphorus concentration in group E rats. Although this decrease lacks statistical significance, the cholesterol concentration of this same tissue shows no change, and therefore the C/P ratio is increased.
ORGAN WEIGHT/BODY WEIGHT RATIOS.

**Rabbits:** The adrenal weight/body weight ratio is increased by 221% ($P<0.001$) in the cholesterol-in-ether fed rabbits compared with the ether controls (groups D-E). This confirms the report of Weinhouse and Hirsch (23) for this same determination. This increased ratio is to be expected on the basis of the marked increase in the cholesterol concentration of the adrenals of cholesterol-fed rabbits, previously discussed.

**Rats:** The adrenal weight/body weight ratio is decreased by 12% ($P<0.05$) in the cholesterol-in-ether fed rats compared with the ether controls (groups D-E). There are no reports in the literature on the adrenal weight/body weight ratio in cholesterol-fed rats. The decreased concentration of plasma cholesterol in the cholesterol-in-ether fed rats could possibly be reflected by a decrease in the adrenal weight/body weight ratio in these animals, inasmuch as there is a relative deficiency of circulating cholesterol, the latter serving as precursor for the adrenal cortical hormones (74).

The adrenal weight/body weight ratio is increased by 17.5% ($P<0.05$) in rats fed a diet pretreated with ether when these are compared with their normal controls (groups E-A). The possibility that rats maintained on a diet pretreated with ether suffer from a deficiency in tocopherol has been mentioned previously in the present report. This situation may be the basis for the present finding, viz., that such
animals exhibit an increased adrenal weight/body weight ratio. A deficiency in tocopherol might represent a "stress" which would result in increased production of adrenal corticosteroid hormones.
V CONCLUSIONS

The conclusions drawn from the present study are as follows.

**Rabbits:** (1) The employment of ether as a solvent for dietary cholesterol does not affect the absorption of cholesterol; (2) the free cholesterol content of aorta, heart muscle, plasma and adrenal is increased in rabbits fed cholesterol-in-ether. The esterified cholesterol content of the aorta, heart muscle and plasma of such rabbits is increased; (3) the lipid-phosphorus concentration in the plasma of rabbits fed cholesterol-in-ether is increased; (4) the C/P ratios are increased in the aorta, heart muscle and plasma of rabbits fed cholesterol-in-ether; (5) the adrenal weight/body weight ratio is increased in rabbits fed cholesterol-in-ether; (6) pretreatment of the basic diet with ether results in an increased concentration of cholesterol in the adrenals, and a decreased concentration of cholesterol in the aorta of the rabbit.

**Rats:** (1) There is an apparent impairment in the absorption of dietary cholesterol when the latter is dissolved in ether; (2) the concentration of esterified cholesterol is decreased in the heart muscle of rats fed cholesterol-in-ether. The concentration of free cholesterol is decreased in the plasma of such rats; (3) there is a decrease in the adrenal weight/body weight ratio in rats fed cholesterol-in-ether; (4) pretreatment of the basic rat diet with ether
results in decreases in the concentrations of free cholesterol in the adrenal and plasma, and of esterified cholesterol in the aorta of the rat; (5) there is a decreased concentration of lipid-phosphorus in the aorta, adrenals and plasma of rats fed a diet pretreated with ether; (6) the C/P ratios in aorta, heart muscle, plasma and adrenal are increased in rats maintained on a diet pretreated with ether; (7) the adrenal weight/body weight ratio is increased in rats whose diet is pretreated with ether; (8) there are species-determined differences in cholesterol absorption, tissue concentrations of cholesterol and lipid-phosphorus and organ weight/body weight ratios in rabbits and rats maintained on cholesterol-in-ether-supplemented diets.
VI SUMMARY

This study was undertaken as an extension of comparative studies, previously carried out in this laboratory, on the oxidative metabolism of aorta and heart muscle from cholesterol-fed rabbits and rats. The present investigation was undertaken for the following reasons: (1) to aid in the interpretation of data accruing from a study of the oxidative metabolism of aorta and heart muscle from rabbits and rats fed dietary supplements of cholesterol and cottonseed oil; (2) to compare the analytical data for free and esterified cholesterol and lipid-phosphorus content of adrenal, aorta, heart muscle and plasma from rabbits and rats fed cholesterol and cottonseed oil diets with those fed cholesterol without a carrying agent; (3) to present uniform experimental and analytical methods for the two foregoing studies, with regard to the strain and sex of the animals, diet, dietary supplements, and laboratory conditions for simultaneous analyses of free and esterified cholesterol and lipid-phosphorus; and (4) to obtain experimental data for the various tissue analyses from the rat, a species refractory to cholesterol per se as an atherogenic stimulus.

Male rabbits and rats, of the New Zealand White and Wistar strains, respectively, were each divided into three experimental groups based upon dietary supplements:

A — normal, no supplement (controls); D — 1% cholesterol in 10% ether; E — 10% ether (controls). For the latter two groups, the ether was immediately evaporated after the
supplements were mixed with the basic diet. Rabbits were maintained on the indicated regimen from 5 to 6 weeks, and rats for 8 weeks. All animals were starved from 20 to 21 hours prior to sacrifice. The indicated tissues were analyzed for cholesterol by the Sperry-Schonheimer method, and for lipid-phosphorus by the Youngburg and Youngburg modification of the Fiske and SubbaRow technique. In addition to the analyses, the total cholesterol/lipid-phosphorus (C/P) ratio was determined for all tissues under study, and the adrenal weight/body weight and heart weight/body weight ratios were calculated for all animals. The data are presented as mean values together with the standard deviation, and standard error of the means. A given pair of mean values for the various dietary groups are compared on the basis of percent difference. Statistical significance is considered attained when the probability value for the difference between any pair of means is 5% or less. The results are as follows.

RABBITS.

Groups E-A comparison: Esterified cholesterol is present in greater concentration in the adrenals, and lesser concentration in the aortas of group E rabbits than in their controls. The data presented for lipid-phosphorus concentration, cholesterol/lipid-phosphorus ratio, and for adrenal weight/body weight and heart weight/body weight ratios fail to reveal any significant changes upon comparison of these
two dietary groups.

Groups D-E comparison: 1. Cholesterol - the concentrations of free and of esterified cholesterol are increased in all tissues studied in group D rabbits, with only one exception: esterified cholesterol in the adrenals, which shows no significant change.

2. Lipid-phosphorus - the concentration of lipid-phosphorus is increased in the plasma of group D rabbits. The remaining tissues are without significant change in lipid-phosphorus concentration.

3. Cholesterol/lipid-phosphorus (C/P) ratio - the C/P ratio is increased in the aorta, heart muscle and plasma of group D rabbits. There is no significant change in this ratio in adrenal tissue.

4. Organ weight/body weight ratios - the adrenal weight/body weight ratio is increased, while the heart weight/body weight ratio is without significant change, in group D rabbits.

RATS.

Groups E-A comparison: 1. Cholesterol - there is a decrease in concentration of free cholesterol in the adrenals and plasma, and of esterified cholesterol in the aortae, of group E rats.

2. Lipid-phosphorus - the concentration of lipid-phosphorus is decreased in the adrenals, aortae and plasma, but is without change in the heart muscles of group E rats.
3. Cholesterol/lipid-phosphorus (C/P) ratio - the C/P ratio is increased in group E rats for all tissues studied.

4. Organ weight/body weight ratios - the adrenal weight/body weight ratio is increased, but the heart weight/body weight ratio is unchanged in group E rats.

Groups D-E comparison: 1. Cholesterol - there is a decrease in concentration of esterified cholesterol in the heart muscles, and of free cholesterol in the plasma of group D rats.

2. Lipid-phosphorus - the concentration of lipid-phosphorus in all tissues studied is without change in group D rats.

3. Cholesterol/lipid-phosphorus (C/P) ratio - the C/P ratio of all tissues analyzed is without change in group D rats.

4. Organ weight/body weight ratios - the adrenal weight/body weight ratio is decreased, but the heart weight/body weight ratio is unchanged for group D rats.

These results, and the conclusions drawn therefrom, are discussed in the light of relevant literature.
VII LITERATURE CITED


(17) Hueper, W.C. The etiology and the causative mechanism of arteriosclerosis and atheromatosis. Medicine, 20: 397, 1941.


(52) Dakin, H.D. Comparative studies of the mode of oxidation of phenyl derivatives of fatty acids by the animal organism and by hydrogen peroxide. J. Biol. Chem. 4: 419, 1908.


(55) Laboratory Animal Care and Feeding. Ralston Purina Company, St. Louis 2, Missouri.


(57) Rachford, B.K. The influence of bile on the fat-splitting properties of pancreatic juice. J. Physiol. 12: 72, 1891.


(73) Wortman, B. Lipidosis on the oxidative metabolism of rat and rabbit ventricular slices. Ph.D. Dissertation. The Ohio State University, Columbus, 1955.


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