analysis in single determinations was different from the morphological classification in one case, which was probably due to an analytical error.

2) The F_2 plants segregated into two categories—one high in DBC and lysine (Hily) and one low (Lowly), both being distributed over the entire protein range from 11 to 22 percent (Table 2). The DBC per 60 mg of protein is negatively correlated with crude protein content equally in both categories.

3) In two crosses the ratio between plants high and low in DBC was not significantly different from 1 : 3, and in another two the same ratio differed significantly from 1 : 3.

4) DBC segregated independently of protein content and the naked and the erectoid characters.

5) From a comparison of the Hily and Lowly plants in the F_2, no regular differences with respect to the variation of time of ripening, straw length, number of tillers, and the number of seeds set could be ascertained. The Hily plants are considerably superior to Hiproly with respect to the last character (Table 2). The seed is smaller in the Hily than in the Lowly plants, but variation in both groups is considerable. There is no strongly expressed tendency for a negative correlation between high DBC (lysine) and yield in the F_2 material.

6) The DBC measurements were verified by amino acid analyses. Of 46 F_2 plants analyzed, two were erroneously classified by DBC determination, which is likely to be due to experimental error. Hily plants in the F_2 generation have an amino acid composition very close to Hiproly (Table 3), whereas the Lowly plants are comparable to the high-yielding, low-lysine paternal parent.

7) Six hundred F_3 seeds from F_2 plants that had amino acids analyzed were studied further with the histological screening technique in a blind test with a reference material. All Hily and Lowly lines were correctly classified. A wide range of morphological variation was recorded among the Hily and Lowly material. The adhesion of the starch grains to the matrix proteins is the critical Hily character. This character displayed a 1 : 3 segregation in the heterozygous F_2 plants in all crosses. The crosses with the ratio deviating from 1 : 3 in the DBC test of yields from F_2 plants (point 3 above) were hence not verified in the F_3 seeds. Hily lines were found that had a much larger proportion of small to large starch grains. Both starch grain types were undeformed.

Thus, the Hily character is due to a recessive gene or linked gene complex. In addition, a number of genes modify seed structure without interfering with the expression of the Hily gene as reflected by the amino acid composition.

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Elevation of Aortic Proline Hydroxylase: A Biochemical Defect in Experimental Arteriosclerosis

Abstract. *The relation of collagen synthesis to experimentally induced arteriosclerosis was studied by measuring proline hydroxylase activity. Gross aortic plaques were produced in rabbits by daily injection of epinephrine (intravenous) and thyroxine (intraperitoneal) for 4, 9, or 14 days. Activity of proline hydroxylase was significantly increased after 4 days of treatment and reached a peak, five- to sixfold increase, after 14 days of treatment. The increase in enzyme activity was correlated with the severity of observed arteriosclerosis. Increase in proline hydroxylase activity may be a possible biochemical defect in the aortas of rabbits with arteriosclerosis induced by injury.*

There is little information on the role of the arterial wall in atherogenesis. The connective tissue of the aortic wall was formerly regarded as metabolically inactive, but it has recently been shown to be an active tissue which responds to external and internal influences (*1*). Collagenous material, which is abundant in both medial and intimal regions of blood vessels, is a dominant component of human atherosclerosis (*1*). Tissue repair mechanisms have been implicated in various model systems of experimental atherosclerosis (*2*). Because accelerated collagen synthesis is a characteristic of healing wounds (*3*), we initiated studies on the collagen synthetic pathway in diseased aortic tissue. Proline hydroxylase activity was measured as a parameter of

Table 1. Proline hydroxylase activity in rabbit aortas after injection of thyroxine (Thy) (0.050 mg/kg for 14 days, intraperitoneally) or epinephrine (Epi) (0.025 mg/kg for the first 5 days and 0.050 mg/kg thereafter, intravenously), or both (Epi-Thy). The rabbits were killed at 4, 9, or 14 days after the beginning of the treatment. Enzyme activity is expressed as the mean (± S.E.) of the amount (dpm) of $[^3H]H_2O$ formed from [3,4-^{3}H]proline per milligram of protein per 30 minutes. Student's *t*-test was used to test for differences between activity in control and treated mice. Numbers in parentheses are the number of determinations.

Treatment	Proline hydroxylase activity		Number of rabbits with lesions graded
	Thoracic aorta	Abdominal aorta	0-1-2-3-4
	4 days		
Control	3896 ± 686 (7)	1422 ± 284 (7)	5-3-0-0-0
Epi-Thy	7064 ± 894 (7)*	2109 ± 273 (8)†	2-4-2-0-0
	9 days		
Control	3722 ± 490 (8)	1553 ± 274 (8)	5-3-0-0-0
Epi-Thy	8943 ± 1526 (9)*	4835 ± 1218 (9)‡	3-2-1-2-1
	14 days		
Control	2750 ± 325 (8)	1637 ± 130 (7)	6-2-0-0-0
Epi-Thy	14179 ± 770 (6)§	5661 ± 637 (6)§	1-2-0-1-2
Epi	4670 ± 1240 (6)†	2026 ± 363 (6)†	3-3-0-0-0
Thy	5659 ± 1050 (8)‡	2678 ± 316 (7)*	2-5-1-0-0

* $P < .01$. † $P > .05$. ‡ $P < .05$. § $P < .001$.

collagen synthesis on the basis of reports implicating this enzyme as a controlling factor in collagen synthesis (*4*).

Gross aortic plaques were induced in male New Zealand rabbits by daily injections of epinephrine and thyroxine for either 4, 9, or 14 days. Epinephrine (0.025 mg/kg for the first 5 days; 0.050 mg/kg thereafter) was injected intravenously with an infusion pump; thyroxine (0.050 mg/kg) was injected intraperitoneally (*5*). Animals were killed by cervical dislocation 24 hours after the last injection. The aortas were quickly removed and chilled, and the degree of aortic disease was evaluated on the 0 to 4 grading system described by Lorenzen (*5*). Aortas were sectioned into thoracic and abdominal segments at the coeliac artery and homogenized in nine volumes of 0.25*M* sucrose in a Polytron ST-10 system.

Proline hydroxylase activity in the aorta was measured in the 15,000*g* supernatant by a method based on the stoichiometric formation of [^{3}H]water and [^{3}H]hydroxyproline when a substrate consisting of a polypeptide rich in [3,4-^{3}H]proline was incubated with enzyme and cofactors (*6, 7*). The presence of proline hydroxylase and its cofactor requirements in the crude supernatant fraction of aorta has been documented (*7*). To rule out the possibility that proteolytic activity in the tissue caused the release of labeled proline from the substrate with subsequent release of [^{3}H]water, crude aorta supernatants were incubated with 0.5 μc of free [3,4-^{3}H]proline or unlabeled proline in the presence and absence of substrate. The crude enzyme was not capable of hydroxylating free proline. Further, the addition of cold proline ($5 \times 10^{-4}M$) had no effect on the enzymatic release of [^{3}H]H_2O from substrate. Incubations were carried out under air in the presence of 0.5 ml of substrate [350,000 disintegrations per minute (dpm)], 7.5 μmole of ascorbic acid, 0.9 μmole of α-ketoglutarate, 0.45 μmole of ferrous ammonium sulfate, 0.5*M* tris(hydroxymethyl)aminomethane-hydrochloride (*p*H 7.5), and enzyme from the aorta (*7*). Tritiated water was collected by vacuum distillation, and the radioactivity was counted in a liquid scintillation spectrometer.

Simultaneous injection of epinephrine and thyroxine produces gross aortic plaques in rabbits which are similar to those found in humans with arteriosclerosis (*8*). We found small focal plaques (grades 1 and 2), representing the early stages of the arteriosclerotic processes, in the thoracic aortas of rabbits treated for 4 days. Rabbits injected with epinephrine and thyroxine for 9 to 14 days exhibited more extensive development of plaques characterized by the presence of large plaques in either the thoracic aorta (grade 3) or in the thoracic and abdominal aorta (grade 4). Our results confirm the observation of Lorenzen (*5*) that both the number of animals exhibiting arteriosclerotic changes and the severity of the changes is dependent on the length of the treatment with epinephrine and thyroxine.

Proline hydroxylase activity of thoracic and abdominal aortas was assayed to determine the degree of correlation with formation of arteriosclerotic plaques (Table 1). A significant increase in aortic proline hydroxylase activity was observed in the thoracic aorta after 4 days of treatment with epinephrine and thyroxine and in both the thoracic and abdominal aortas after 9 or 14 days of drug treatment. Proline hydroxylase activity increased in both the thoracic and abdominal aortas as the period of drug treatment was increased. After 14 days of treatment thoracic proline hydroxylase activity increased five- to sixfold and the abdominal enzyme activity increased three- to fourfold. Plaque formation induced by epinephrine and thyroxine occurs primarily in the thoracic aorta, the area which accounted for the largest quantitative increase in proline hydroxylase. The severity of plaque formation and the magnitude of the increase in proline hydroxylase are both related to the duration of drug treatment.

Aortas from rabbits injected with only epinephrine or thyroxine for 14 days did not exhibit either the severe arteriosclerotic plaques (that is, grades 3 and 4) or large increases in proline hydroxylase activity as seen with simultaneous injection of epinephrine and thyroxine (Table 1). Administration of epinephrine or thyroxine for 14 days resulted in an approximate twofold increase in thoracic aorta proline hydroxylase activity; simultaneous administration of the drugs for the same period of time resulted in a five- to sixfold increase. Therefore, the observed increases in arteriosclerotic disease and proline hydroxylase activity are a metabolic consequence of the aortic injury induced by the simultaneous injection of epinephrine and thyroxine.

Takeuchi *et al.* (*9*) have reported increased proline hydroxylase activity in the liver of rats with hepatic fibrosis induced by carbon tetrachloride. These authors observed a fivefold increase in liver enzyme activity, but, because of problems related to nonlinearity of their assay system with highly active samples, they suggested that the true increase was even greater. Our system reached substrate limitation with the highest values reported (14-day group). Thus, the true magnitude of the increase in aortic proline hydroxylase activity may be in excess of the limitations imposed by our system.

On the basis of histochemical studies, Gresham *et al.* (*10*) suggested that early atherosclerotic plaques of man, turkey, rat, and rabbit are active sites of collagen synthesis. Other investigators have reported correlations between the content of proline and hydroxyproline in the aorta and atheromatosis of the aorta (*1, 11*). Our results provide more direct evidence that collagen synthesis increases during arteriosclerotic plaque formation in the rabbit; that the increase can be detected in the early stages of plaque development (that is, 4 days); and that synthesis continues to increase as the disease progresses.

In these experiments the degree of arteriosclerosis was determined by visual grading methods, which prevented a precise evaluation of the extent of histological damage to the aorta in individual animals. We cannot infer from our data that the elevation of proline hydroxylase activity is an initiating event in plaque formation as opposed to a secondary response involving a repair mechanism, even though varying increases in enzyme activity were observed in all animals receiving treatment with epinephrine and thyroxine. However, our results are significant in view of the suggestion made by Hovig *et al.* (*12*) of the importance of platelet adhesion to extracellular collagen in the formation of thrombi from free-flowing blood. We agree with an earlier suggestion (*4*) that the hydroxylase for collagen should be vulnerable to specific inhibition.

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Phosphonolipids: Localization in Surface Membranes of Tetrahymena

Abstract. *Approximately 60 percent of the phospholipids from the membrane sheath of* Tetrahymena pyriformis *cilia contain 2-aminoethylphosphonic acid. This is more than twice the concentration found in total cell lipids. The resistance of these lipids to hydrolytic enzymes suggests that they increase the stability of the surface membranes.*

While the ester linkage is the most common mode of bonding phosphorus to carbon in nature, phosphonic acids, which contain direct carbon-to-phosphorus bonds, have been found in a variety of organisms (*1*). Compounds containing these bonds are generally structural analogs of naturally occurring phosphate esters. The most widely distributed phosphonic acid is 2-aminoethylphosphonic acid (AEP), an analog of phosphorylethanolamine. 2-Aminoethylphosphonic acid occurs bound to proteins and lipids as well as in the free form and sometimes accounts for as much as one-half of the total phosphorus of the organism (*1*).

The carbon-phosphorus linkage is highly resistant to hydrolytic enzymes (*2*) and, in addition, the presence of AEP in phospholipids prevents their degradation by certain phospholipases (*3*). A possible physiological role for such lipids, termed phosphonolipids, has been suggested by Kittredge and Roberts (*1*) and by LaNauze (*4*). They postulated that the resistance of phosphonolipids to enzymatic breakdown may confer stability on the cellular membranes of which these molecules are a part. Such lipids might provide greater integrity to the outer membrane, especially where that membrane encounters hydrolytic enzymes, as in the case of rumen protozoa.

Our studies on membrane fractions from *Tetrahymena pyriformis*, a protozoan rich in AEP, have provided data which support suggestions of a protective role for the compound. We report here that surface membrane fractions of *Tetrahymena,* particularly membranes surrounding the cilia, contain higher percentages of phosphonolipids than do membranes within the cell.

Logarithmic phase cultures of *Tetrahymena pyriformis*, strain E, grown in 200-ml lots in enriched proteose-peptone medium (*5*) were deciliated according to the procedure of Rosenbaum and Carlson (*6*), modified so that suspensions of deciliated cells were diluted with only 2 volumes of growth medium instead of the 20 volumes used previously (*6*). Deciliated cell suspensions were centrifuged for 10 minutes at 650*g* and 2000*g* to remove whole cells and cell fragments, respectively. Cilia were then collected by centrifugation at 16,000*g* for 15 minutes. Electron micrographs showed that almost all detached cilia retained their membrane sheaths (Fig. 1) and that about 80 percent of the membranous material in the isolated fraction was associated with the cilia. Membranous contamination was due primarily to the presence of mitochondria arising from the few cells ruptured during the procedures. Ciliary phospholipids accounted for approximately 2.0 percent of the total cellular lipid phosphorus.

Table 1. Cellular distribution of lipid phosphonate in *Tetrahymena*. Figures in parentheses indicate number of experiments averaged.

Fraction	Phosphonate (% of total lipid phosphorus)	Average deviation
Whole cells	29.0 (4)	5
Crude mitochondria	32.2 (2)	0.5
Crude microsomal fraction	29.8 (3)	2
Cilia	63.0 (6)	7

Deciliated cells were disrupted in a Potter-Elvehjem homogenizer in 0.2*M* potassium phosphate buffer, *p*H 7.1, containing 0.1*M* NaCl and 3 m*M* ethylenediaminetetraacetate. Homogenates were centrifuged at 19,600*g* for 20 minutes to obtain crude mitochondria. The resulting supernatant was centrifuged at 100,000*g* for 60 minutes to obtain a microsomal fraction.

Lipids were extracted according to the method of Bligh and Dyer (*7*) and chromatographed on thin-layer silicagel G plates as described (*3, 5*). In labeling experiments, radioactivity in lipid fractions scraped from thin-layer chromatography (TLC) plates was assayed with a Packard Model 3310 scintillation spectrometer. Total phosphorus in lipid extracts was measured by the method of Bartlett (*8*) modified by digestion with perchloric acid according to Marinetti (*9*). Ester phosphorus was determined by the method of Aalbers and Bieber (*10*). Phosphonate phosphorus was calculated as the difference between total and ester phosphorus.

In repeated trials, 50 to 75 percent of the phosphorus in ciliary lipids was found as phosphonates (Table 1). The percentage of phosphonate phosphorus in whole cells and in isolated mitochondria and microsomes was 25 to 35 percent. Isolated pellicles seem to contain slightly higher amounts of AEP—up to 40 percent (*11*).

These findings were based on differential analyses of total lipid extracts, as described above. While such data do not imply which phosphonolipid is enriched, evidence on this point is provided by visual examination of TLC plates. In the case of cilia extracts, spots previously identified as 2-aminoethylphosphonolipid (*3*) were much darker, relative to other lipids, than in extracts of other membranes (Fig. 2) indicating a higher concentration of this compound.

The finding that phosphonolipids are resistant to degradation by lipolytic enzymes (*3*) suggested that the observed high percentage of phosphonate in ciliary lipids might be at least in part the consequence of selective breakdown