In Vitro Study of Cholesterol Metabolism in the Calf Aorta

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The calf's aorta has been maintained in vitro for prolonged periods under aseptic conditions. Studying the aorta's function as regards cholesterol biosynthesis, accumulation and release, under the experimental conditions of hyper and normal blood pressure, estrone administration, and dilutions of serum have led to the conclusion that cholesterol metabolism can be markedly altered by these conditions and that there may be factors or systems normally present in blood which regulate lipid metabolism in the organ.

It has been shown that the excised calf aorta was capable of performing two functions, possibly unrelated, in an in vitro system. The first of these was that of accumulating cholesterol as determined by measuring the concentration of cholesterol in the aorta before and after the experiment. The second capacity was that of synthesizing cholesterol from acetate. This function was determined by observing the incorporation into cholesterol of C14 from carboxyl labeled acetate. The latter finding corroborated earlier findings by Siperstein, Chaikoff and Cherniek, and those of Schwenk and Werthessen.

The most important point delineated by the earlier studies was the relationship seen between glucose consumption and cholesterol accumulation. In 72 hour experiments it was observed that the degree of cholesterol accumulation found in the aorta was directly related to the glucose consumption per gram net weight of the organ. These data indicated that cholesterol accumulation by the aorta could be an energy dependent activity. We have directed our work toward extending these studies.

This communication reports the effects of pressure, estrone and dilutions of serum on this isolated system.

METHODS

The methods employed have been the same as those described in the earlier communication except for certain improvements in technic leading to greater accuracy.

Briefly recapitulated, an aorta is taken from a calf weighing approximately 100 pounds; it is stripped of all adventitious tissue and with its afferent arteries ligated, is set up and maintained in vitro in a system which approximates to some degree its situation in vivo. Perfusion, usually consisting of 50 per cent defibrinated bovine blood and 50 per cent White's solution, carrying normal O2 and CO2 tensions, enters the aorta's lumen through a cannula inserted into the remaining portion of the arch. The perfusion pressure is conveniently regulated by adjusting the driving mechanism. The system is completely aseptic in the sense that no microorganisms grew within it in any of the reported experiments. Typically the aorta weighed about 20 Gm., wet weight, and was nourished by 500 ml. of perfusate. The blood employed never exceeded 7 days of age. Improvements in technic now permit the setting up of 95 out of 100 experiments without contamination. They also permit the measurement, with less than a 1 per cent error, of the volume of perfusate. In addition, a considerable number of developments in the technics of analysis for cholesterol titre and the degree of isotope accumulation permits more rapid and more precise measurement of these parameters. These latter improvements are to be reported separately.

Aliquots of the aorta taken before and after the experiment permit determination of the cholesterol concentration in the wall. Measured quantities (as milliliters) of C14 labeled acetate are added to the perfusate to follow biosynthesis. Isolation of the cholesterol from the aorta and perfusate followed...
by the determination of the isotope content of the cholesterol permit measurement of the degree of biosynthesis which occurred. The various experiments reported below can be compared, one with another, providing that the amount of labeled carbon added to each is known with precision.

As shown in the earlier publication, there is a cholesterol concentration gradient over the aorta's length. The concentration at the "arch" is lower than that at the bifurcation. On a statistical basis the average of an aliquot taken from near the heart and one taken at the bifurcation is representative of the total. This value is referred to in the text as \( C_0 \), i.e., the original concentration or cholesterol. To determine the change in cholesterol concentration a strip of aorta the full length of the perfused segment is taken, cut in two, and the concentration of cholesterol in each portion determined. The average of these portions provides the final concentration level. This value is referred to in the text as \( C_2 \). \( \Delta C \), or the change in concentration, is equal to \( C_2 - C_0 \).

To determine biosynthesis, or as it should be phrased more accurately for this report, the degree of isotope incorporation of \( ^{14}C \)-acetate into cholesterol, use is made in these studies of the fact that throughout all the studies 500 ml of perfusate was employed. A known dosage of acetate was added to each perfusion. To compensate for slight unavoidable changes (approx. 10 per cent) in the dosage, a standard of reference (.0593 milli-curies) was employed. Consequently, to compare various experiments as to the degree of isotope incorporation, it was only requisite to determine the isotope content of the cholesterol and to correct the observed value by the ratio of the used dosage to the standard dosage.

To express the results, the symbols \( TC_a \), total count per minute of the cholesterol in the aorta, \( TC_p \), total count per minute of the cholesterol in the perfusate, and \( TC_s \), total count per minute of the system, are employed for brevity. The total count value is employed to compensate for the fact that these experiments deal with organs that are (1) of different weights, (2) contain different initial concentrations of cholesterol and (3) accumulate different amounts of cholesterol. Attempts to employ the simpler value of the specific activity of the cholesterol as directly observed revealed that even when corrected by the milli-curies of acetate available per gram of aorta, the specific activity showed enormous and erratic scatter.

The total count method of comparison is the best devised to date. To obtain this value, the specific activity (as count/min./mg.) is multiplied by the total amount (as mg.) of cholesterol present in aorta or perfusate. The total count of the system is the sum of the aorta and perfusate values. Unfortunately, these still show sufficient erratic variation to indicate that additional correction factors to correlate one experiment with another must be sought.

Determinations of the glucose titre of appropriately timed samples of the perfusate permit measurement of glucose consumption by the system.

**Experiments**

*The Effect of "Blood Pressure" on Cholesterol Metabolism*

For this study each experiment was run for exactly 24 hours and the pressure of the perfusate in the lumen of the aorta was set to range, as an average of the diastolic and systolic pressures, as closely to 100 mm. Hg or 200 mm. of Hg as the particular organ would permit. The difference between the systolic and diastolic pressures was regulated to be not less than 10, nor more than 30 mm. of Hg.

The results of the study as regards the high pressure series is shown in figure 1. It demonstrates the relationship which exists between \( C_0 \) (the original concentration of cholesterol in the aorta) and \( \Delta C \) (the difference between the final concentration \( C_2 \) and \( C_0 \)). This figure shows clearly that the degree of change of cholesterol concentration (\( \Delta C \)) is affected by the concentration at the beginning of the experiment. The inverse nature of the relationship permits the conclusion that within the experimental conditions employed there is a limit
to the amount of cholesterol that an aorta can accumulate. Extrapolation to the intercept shows that this limit lies in the neighborhood of 5 mg. of cholesterol per gram dry weight of aorta.

The data for the low pressure series, plotted in the same fashion as for the high pressure series is presented in figure 2. Here, no reasonable arrangement of the data is seen. Thus not only does there appear to be no significant correlation in the low pressure series, but it is also clear that when viewed in this fashion, there is a significant difference between the high and low pressure series of experiments.

In many of the above cited experiments, labeled acetate was added. By examining the degree of isotope incorporation in the aorta, another independent parameter is therefore available for study. Demonstration of a difference in behavior of cholesterol in the aorta as a function of pressure via this independently measured parameter would leave little room for doubt as to the capacity of pressure of the internal fluid, per se, to affect cholesterol metabolism.

The studies on isotope incorporation in the aortic cholesterol have revealed a difference in behavior of cholesterol in the aorta as a function of pressure. By two criteria of measurement it has been possible to show a difference between the low pressure and high pressure series, it seems reasonable to conclude that the internal fluid pressure of an aorta is capable of markedly affecting the overall cholesterol metabolism in that aorta.

![Graph](image)

**Fig. 2.** Relationship at low perfusion pressure (100 mm. Hg) of the change in concentration, ΔC, to the original concentration, C₁, of cholesterol in the aortic wall expressed in mg./Gm. dry weight. Each point represents one perfusion which lasted precisely 24 hours.

<table>
<thead>
<tr>
<th>Perfusion no.</th>
<th>Glucose consumption (mg./Gm. wet weight/24 hours)</th>
<th>Total count per minute of aortal cholesterol (TCa) X 10⁹</th>
<th>Total count per minute of perfusate cholesterol (TCP) X 10⁹</th>
<th>Total count per minute of cholesterol in system (TCo) X 10⁹</th>
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The Influence of Adding Esterone to the Perfusate

Estrone has been studied under hypertensive conditions both in 24-hour perfusions and in 72-hour experiments. The perfusate, except for the addition of estrone, was the standard solution normally employed. The 24-hour experiments were controlled by the high pressure series cited earlier.

The data obtained are presented in table 2. It is clear from inspection of the table and comparison with table 1 that in 24 hours no effect of estrone is apparent. In the 72-hour experiments, however, while ΔC is unaffected, the degree of isotope incorporation has undergone a significant change. By utilizing the total count per minute of the whole system (TCp) as a basis of comparison, it is found that the estrone group presents an average value of 31 X 10^4 counts/min. is found to be significant by t test, p equals approximately 0.03.

Thus, not only is it apparent that the aorta can respond to estrogenic stimulation in vitro, but it also appears possible that this response involves some change in the aorta requiring the passage of time to become evident.

Passage of Cholesterol from Aorta into Perfusate

Six Day Perfusion. The perfusates of 24-hour experiments as shown in tables 1 and 2 contained radioactive cholesterol. This could indicate that newly synthesized cholesterol was moving from the aorta into the perfusate. The experiment designed to test this possibility depended upon the observation, reported in the earlier communication, that even after prolonged periods of perfusion (200 hours) the aorta could synthesize cholesterol from acetate. If synthesis were possible at the end of such a prolonged period of in vitro maintenance, then it should be doing so at all times prior to that established limit.

Accordingly, the experiment was performed as follows: (1) An aorta was placed in the perfusion system for a 6 day period (144 hours), the perfusate used throughout was made up in one large bottle from which 500 ml. aliquots were taken; (2) The perfusate was changed, and a new charge of perfusate of the same volume was added at 24-hour intervals; (3) To each charge of perfusate was added 0.059 millicuries of labeled acetate; (4) Each perfusate was extracted for cholesterol, and the isotope incorporation determined as the count/min./mg.

The data plotted against the day of perfusion are presented in figure 3. It is clear that after 2 days there is a marked daily increment in the C14 titre of the cholesterol in the perfusate. This result is precisely that which would be expected if, as the experiment progressed, the aorta released cholesterol to the perfusate and replaced that cholesterol with material newly-formed from labeled acetate. Under these conditions the isotope titre of the aortal cholesterol would continuously increase and, as a consequence, the cholesterol released into the perfusate would, each day, have a higher and higher titre. This, in turn, would be reflected in the perfusate by an increasing C14 titre in the perfusate's cholesterol.

While the findings described above are in
accord with the hypothesis presented, it is also possible to explain them on different grounds, as, for example, an increasing dissolution of aortal tissue and the concomitant release of more labeled cholesterol each day, rather than essentially the same amount of cholesterol each day, but of a higher and higher C$^{14}$ titre.

The data of this experiment, plus the perfusate findings which led to its undertaking, suggested the critical study of the relationship of the perfusate cholesterol to the aortal cholestrol as reported in the next series of experiments.

Experiments with Dilutions of Serum by White's Solution as Perfusate. The primary objective of this experiment was to ascertain the importance of serum constituents in making possible the movement of cholesterol from the aorta into the perfusate. To prevent confusion, such as could result from biosynthesis of cholesterol by formed elements of the blood, only pure serum and White's solution were used as components. Quite unexpectedly, additional data, of perhaps greater import, were yielded by the study. The experiments were carried out, in all details, in precisely the same fashion as the 11 high pressure runs presented in Table 1, hence the latter were used as a reference point. The C$^{14}$ values of the aortas used ranged from 2.65 to 5.19. No correction for the regression of ΔC as a function of C$^{14}$ was employed since the control series ranged from 2.92 to 4.32, and the variance encountered was not sufficient to obscure the differences.

Figure 4 shows that in the series of serum dilutions employed, namely, 0, 12.5, 25, 50, 75 and 100 per cent, the average ΔC value for the dilutions changes markedly. The lowest values are found at the 0 and 100 per cent serum levels. The highest ΔC average value is found at the 12.5 per cent dilution level as well as the greatest variation around the average. From this dilution to the 100 per cent serum concentration, both the variation and the average ΔC levels decrease. The ΔC values for the 12.5 and 25 per cent levels have been compared by the t test with the high pressure reference controls utilizing 50 per cent diluted blood. The average ΔC of 1.58 is significantly different at a probability level of less than .01 from the 0.14 value of the high pressure series.

Thus, it is clear that a serum concentration and a concomitant perfusate cholesterol concentration of 25 per cent or less, but not 0, is conducive toward one of the highest cholesterol accumulation levels that has been observed.
CONCENTRATION

Fig. 5. The $^{14}$C labeled cholesterol content of aortas and perfusates as a function of the dilution of serum employed in the perfusate. The solid circles represent the values obtained for each experiment. The large open circles represent the average values. The numerical values as expressed are reduced by the factor of $10^4$.

It should be particularly noted that the comparisons just made employed as a reference base red cell containing perfusate. The serum content of the reference base series was approximately 50 per cent. It is therefore possible to state that the red cells are not a limiting factor as regards degree of cholesterol accumulation.

In figure 5 is plotted the total radioactivity of the cholesterol in the perfusate. The change in isotope content is not considerable over the range of serum dilutions. It should be emphasized at this point, however, that the value of 0 given for the 0 per cent serum perfusate is as close to 0 as our techniques can measure. From both the total count in the aorta ($T_C$) and the total count in the perfusate ($T_P$) data presented in figure 5, it is obvious that $^{14}$C from acetate incorporation into cholesterol is markedly altered as the composition of the perfusing medium shifts from 100 per cent White's solution to 100 per cent serum. The amount of $^{14}$C from acetate incorporated into cholesterol varies inversely to the concentration of serum in the perfusate. Except for the 0 per cent serum value, the relationship is the same as for the accumulation of cholesterol.

A statistical comparison between the high pressure reference series and the serum studies reveals two significant differences. The first of these is found when the values for the total aortal cholesterol count of the 75 per cent and 100 per cent dilutions are compared to the controls. The average for the two serum dilutions is $81 \times 10^2$ as against $144 \times 10^2$ for the controls. The difference of $63 \times 10^2$ is statistically significant at a probability level of 0.03. Contrariwise, the average value of $283 \times 10^2$ for the 12.5 per cent and 25 per cent levels is significantly greater than the control value with a probability level of 0.04. Thus, at low serum levels, the isotope incorporation is greater than in the controls, whereas, at the high serum levels it falls far below.

The interrelation of $T_C$ and $T_P$ and per cent serum is apparent from figure 6. Here are plotted against the dilutions of serum the ratio $T_C/TC$, as well as the ratio $T_P/TC$. The fit of these points to the curves drawn through them is remarkable in view of the variance demonstrated in the two other figures. The inference from this figure is that some component of serum tends to increase the movement of newly synthesized cholesterol from the aorta into the perfusate.

Briefly, then, in all three of the cited figures, the aorta's performance changed as the serum

\*The total perfusate was extracted and the total extract was tested by both radioactivity and the Lieberman-Burchard reaction for the presence of cholesterol. None was observed.
content changed, from 0 per cent to 100 per cent. In two of the figures, (4 and 5), the observed values decreased as 100 per cent serum was approached (except for ΔC at 0 per cent serum, fig. 4). Not only was there a reduction in the degree of activity observed, but there was a reduction in the variance of the values as 100 per cent serum was approached. Therefore, the conclusion seems inescapable that as regards cholesterol biosynthesis and cholesterol accumulation, the dilution of serum involves the diluting out of a controlling factor.

The final figure (6) shows the dependence of the amount of labeled cholesterol in the perfusate on the concentration of serum in the perfusate as a positive relationship. It seems reasonable, therefore, to conclude that the movement of cholesterol from the aorta into the perfusate, as well as its biosynthesis and extent of accumulation in the organ, is dependent on controlling factors contained in serum.

**Discussion**

The results of the experiments cited above permit certain generalized conclusions when viewed in toto.

The 24 and 72 hour experiments with estrone not only indicate that estrone has an effect on aortic metabolism, but also show that time must elapse before the effect is clearly evident. The necessity of such a time lapse can only be taken to mean that the effect observed is secondary to primary changes induced by the estrogen. Consequently, the conclusion is possible that the aorta not only can respond to estrogenic stimulation but that in so doing there must occur alterations on an enzymic level within the organ.

The response of the aorta to estrone, the dependence of manner of cholesterol accumulation on hypertension and, finally, the dependence of the degree of isotope incorporation into cholesterol on pressure were established by these data. These three findings must mean that the behavior or metabolism of cholesterol in the aorta is controllable. Of primary significance is the fact that these three findings were made under conditions wherein the cholesterol titre of the perfusing medium was essentially 50 per cent of normal. Yet, at this low level of hypocholesterolemia, aortae were seen to almost double their content of cholesterol within a 24-hour period.

The conclusions drawn from the serum dilution studies provide further evidence of control of cholesterol levels in the aortae that are independent of cholesterol concentration in the blood. The nature of these agents is not indicated by the serum dilution data. They may be physical, physicochemical or hormonal in nature. That they may operate through control of biosynthesis and removal of synthesized cholesterol from the aorta is also evident from the data.

The general conclusion that the aortic content of cholesterol and its rate of biosynthesis of the lipid is subject to control may be the most important inference from the study. Lack of control of a metabolic function is a frequent primary factor in the etiology of a number of chronic diseases. Only further study can reveal its importance in atherosclerosis.

**Summary**

Data obtained from the calf aorta perfused in vitro under aseptic conditions with variations in pressure and perfusate composition and with estrone added to the perfusate support the following conclusions:

At a pressure of 100 mm. Hg., the aortic wall incorporated into its cholesterol roughly twice as much C\(^{14}\) from labeled acetate as it did at 200 mm. of Hg. At pressures of 200 mm. Hg, there was an accumulation of cholesterol in the aortic wall that was inversely proportional to the original concentration of cholesterol in the aortic wall. This constant relation to original concentration was not apparent at a pressure of 100 mm. Hg.

Estrone added to the perfusion fluid showed no significant effect in 24 hours, but at 72 hours, isotope incorporation was more than doubled. There was, however, no change in the degree of accumulation of cholesterol in the aortic wall as compared to controls without estrogen.

By using a perfusate free of red cells and by varying the percentages of serum and White's solution used in the perfusion fluid; it was shown that serum contains factors that control the rate of biosynthesis, the concentration of
cholesterol in the aorta's wall and the proportion of newly synthesized cholesterol that moves from the aortic wall into the perfusate.

These findings imply that complicated metabolic processes occur within the aortic wall, that these processes are subject to extra-vascular control, and that they may determine the degree of local accumulation of cholesterol by influencing both its rate of formation in, and its rate of removal from the aortic wall.

SUMMARIO IN INTERLINGUA

Datos obtenite ab le aorta vitellin perfundite in vitro sub conditiones aseptic con variationes de pression e de composition del liquido perfusional e con le addition de estrona al liquido perfusional supporta le sequente conclusiones:

A un pression de 100 mm Hg, le pariete aortic incorporava in su cholesterol circa 100 pro cento plus C M ab acetato etiquettate que illo incorporava a un pression de 200 mm Hg. A pressiones de 200 mm Hg, il habeva un accumulation de cholesterol in le pariete aortic que esseva inversemente proportional al concentration original de cholesterol in le pariete aortic. Iste constante relation al concentration original non esseva evidente al pression de 100 mm Hg.

Le addition de estrona al liquido perfusional non produceva effectos significative post 24 horas, sed post 72 horas le incorporation del isotopo esseva plus que duplate. Tamen, il non habeva un alteration del grado de accumulation de cholesterol in le pariete aortic in comparation con testa de controlo sin estrogeno.

Per usar un fluido de perfusion libre de erythrocytos et per variar le procentage de sero e de solution de White usate in le fluido de perfusion, il esseva possibile monstrare que le sero contine factores que regula le progresso del biosynthese, le concentration de cholesterol in le pariete del aorta, e le proportion del nove-mente synthetisate cholesterol que se transfere ab le pariete aortic a in le fluido de perfusion.

Iste constatationes significa que complexe processos metabolic occurre intra le pariete aortic, que iste processos es regulate per factores extra-vascular, e que illos pote determinar le grado de accumulation local de cholesterol per influentiar tanto su formation in le pariete aortic como etiam su abferimento ab le pariete aortic.

REFERENCES

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