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Cholesterol concentration and lipoprotein pattern in the serum of veal calves fed milk replacers with various levels of cholesterol

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Summary

Veal calves, aged about 1 week, were fed for 146 days milk replacer diets supplemented with various levels of cholesterol. Four groups consisting of 11 or 12 animals received diets to which 0, 0.19, 0.56 or 0.93 % (on the basis of air-dry matter) cholesterol was added at the expense of fat. Cholesterol feeding significantly increased the level of serum cholesterol. This increase was either linear or S-shaped over the entire range of cholesterol feeding, depending on the time during which the calves were fed the diets. In the calves fed cholesterol, the ratio of free to esterified cholesterol in the serum was significantly increased. The ingestion of cholesterol markedly increased the cholesterol content of the VLDL, IDL and LDL fractions in the serum. When compared to the 0.56%-cholesterol diet, the inclusion of 0.93 % cholesterol in the diet did not further increase the cholesterol concentrations in the IDL and LDL fractions. Upon density gradient ultracentrifugation of serum prestained for lipid with Sudan Black, it was observed that dietary cholesterol caused the HDL_1 particles ($1.063 < d < 1.092$) to shift towards a lower density range. Cholesterol feeding effected an increase in the level of serum triglycerides, the increase being already maximal with 0.19 % cholesterol in the diet. Dietary cholesterol induced elevated concentrations of serum phospholipids, the elevation being proportional to the amount of cholesterol in the diet.

Zusammenfassung

Mastkälber, etwa eine Woche alt, wurden während 146 Tagen mit Milchersatzmitteln, die mit verschiedenen Cholesterinmengen angereichert waren, gefüttert. Vier Gruppen, bestehend aus 11 oder 12 Tieren, erhielten Diäten, denen 0, 0,19, 0,56 oder 0,93 % (Gewichtsprozente auf trockener Stoffbasis) Cholesterin – auf Kosten des Fettgehaltes – beigefügt wurden. Cholesterin in der Diät erhöhte signifikant den Serumcholesterinspiegel. Dieser Anstieg war entweder linear oder S-förmig über den gesamten Bereich der Cholesterinzudienung, abhängig von der Versuchsdauer, festzustellen. In den cholesteringefütterten Kälbern war das Verhältnis von freiem zu verestertem Cholesterin im Serum signifikant erhöht. Die Aufnahme von Cholesterin erhöhte deutlich die Cholesterinkonzentration von den VLDL-, IDL- und LDL-Fraktionen im Serum. Bei einem Vergleich der 0,93%igen Cholesterindiät mit der 0,56%igen zeigte sich kein weiterer Anstieg der Cholesterinkonzentrationen der IDL- und LDL-Fraktionen. Nachdem die vorher mit Sudanschwarz gefärbten Lipoproteine des Serums mittels Dichtheitsgradient-Ultrazentrifugation getrennt waren, wurde festgestellt, daß Nahrungscholesterin die Dichte der HDL-Partikel

(1,063 < d 1,092) erniedrigte. Die Zudienung von Cholesterin verursachte keinen Anstieg der Triglyceridkonzentration im Serum; ein maximaler Anstieg wurde bereits mit der 0,19%igen Cholesterindiät erreicht. Nahrungscholesterin induzierte erhöhte Konzentrationen der Serumphospholipide; diese Erhöhung korrelierte mit dem Cholesteringehalt in der Diät.

Key words: dietary cholesterol, calves, serum cholesterol, lipoproteins, serum phospholipids, serum triglycerides

Introduction

In the young bovine, dietary cholesterol has been repeatedly shown to induce high serum cholesterol levels (3–5, 11, 12, 21, 22) and atherosclerotic changes in the arterial tissues (21, 22). We have recently described the effect of a relatively high cholesterol load (1 g/100 g air-dry feed) on the distribution of cholesterol between lipoprotein fractions of the serum of veal calves (5). However, we are not aware of reports describing a dose-response relationship between dietary cholesterol and the concentration of cholesterol in whole serum or in serum lipoproteins of non-ruminating calves. The object of the present study was to investigate in veal calves the time course of the effect of differing dietary cholesterol loads on the level of serum total cholesterol. At the end of the experimental period, the levels of free and esterified cholesterol, triglycerides and phospholipids in whole serum, and the concentration of cholesterol in lipoprotein fractions were also examined.

Materials and methods

Animals and diets

In this experiment, male Dutch Friesian-Holstein calves were used. The calves were purchased at a market at the age of about one week. The initial body weight of the calves was 42.3 ± 3.7 kg (± SD). The calves were housed individually in wooden boxes with slatted floors. The calves were pail-fed twice a day (at 7.00 a.m. and 5.00 p.m.) a reconstituted milk replacer.

On arrival in the calf house, the animals were divided into four comparable groups consisting of 12 calves and for 21 weeks were fed milk replacers containing different levels of cholesterol. The composition of the diets is given in table 1. Cholesterol was added to the diets at the expense of fat. Apart from the cholesterol content, the levels of the analysed components were similar for all diets. The control diet contained 40 mg cholesterol per 100 g, most likely originating from the fat and/or the skim milk powder component.

The milk replacers were reconstituted in hot water. As is common practice, the feeding level was increased from 125 g air-dry feed per meal in the 1st week to 1600 g in the 21st week. During the experiment one animal in the control group died. Growth rates of all dietary groups were similar, the mean body weight being 215.5 ± 16.4 kg (± SD) at 21 weeks.

Analytical methods

At the indicated days (fig. 1), blood samples were taken between 10.00 and 11.00 a.m. from the jugular vein. The samples were collected in tubes without anticoagulant, and after standing at room temperature for at least one hour, serum was prepared by low speed centrifugation at room temperature.

Table 1. Composition of milk replacer diets[1]).

		Cholesterol-enriched diets[2])		
	Control diet	0.19 %	0.56 %	0.93 %
Ingredient		(g/kg)		
Fat blend	194.7	192.8	189.0	185.3
Skim-milk powder	605.2	605.2	605.2	605.2
Whey powder	160.6	160.6	160.6	160.6
Vitamin/mineral premix	39.5	39.5	39.5	39.5
Cholesterol[3])	–	1.9	5.6	9.3
Chemical analysis		(weight %)		
Moisture	3.4	3.4	3.4	3.4
Ash	6.3	6.4	6.3	6.3
Crude protein	21.9	22.3	22.2	22.0
Crude fat	20.5	19.7	19.6	19.8
Carbohydrates[4])	47.9	48.2	48.5	48.5
Cholesterol	0.04	0.25	0.49	0.76

[1]) Supplied by Trouw & Co., N.V., Putten, The Netherlands.
[2]) Diet codes refer to the amount of added cholesterol.
[3]) Purchased from Van Schuppen-Chemicals Veenendaal, The Netherlands.
[4]) Calculated values.

Cholesterol was measured in serum enzymatically according to Röschlau et al. (16) using the kit (peroxidase method) supplied by Boehringer Mannheim GmbH, West Germany. The reproducibility (coefficient of variation) was routinely less than 1.1 %. As cholesterol standards, three calibrated sera with low, medium and high cholesterol concentrations were used; the cholesterol concentration of these sera was determined by the method of Abell et al. (1).

The lipoproteins in pooled samples of serum from each experimental group were separated by ultracentrifugation as described by Terpstra et al. (20). The lipoproteins in the gradient were visualized by prestaining the serum with Sudan Black prior to ultracentrifugation. The various lipoprotein fractions were collected by aspiration. Since the lipoprotein density profile in calves differs from the pattern observed in humans (19), which is used as the basic classification, we have used a modified scheme with slightly different density (d) classes. The following density classes (d in g/ml) were isolated: VLDL ($d < 1.006$); IDL ($1.006 < d < 1.019$); LDL ($1.019 < d < 1.063$); HDL_1 ($1.063 < d < 1.092$); HDL_2 ($1.092 < d < 1.125$); HDL_3 ($1.125 < d < 1.210$); and the bottom fraction ($d > 1.210$)[1]). Cholesterol in the lipoprotein fractions was estimated by the catalase method of Röschlau et al. (16).

Serum-free and esterified cholesterol were determined enzymatically according to Röschlau et al. (16), using the kit (catalase method) supplied by Boehringer Mannheim GmbH, FRG. The concentration of lipid phosphorus was measured in a lipid extract of the serum (10) as described by Bartlett (2) and modified by Böttcher et al. (7). Serum triglyceride-glycerol concentrations were determined enzymati-

[1]) Abbreviations: VLDL = very-low density lipoproteins, IDL = intermediate density lipoproteins, LDL = low density lipoproteins; HDL = high density lipoproteins.

cally after saponification with ethanolic KOH (9). Cholesterol in the diets was assessed by gas-liquid chromatography (13) of the non-saponifiable fraction.

Results

As table 1 shows, the analysed amounts of cholesterol in the 0.19, 0.56 and 0.93%-cholesterol diets did not agree well with the added amounts. The reason for this discrepancy is not clear. The gas-liquid chromatographic determination of cholesterol was found to be unbiassed and reproducible (the coefficient of variation was less than 5 %), and the added cholesterol, as judged from the gas chromatogram, was very pure.

Figure 1 illustrates the time course of the concentration of serum total cholesterol in the veal calves. In the control calves, the level of serum cholesterol increased from 0.96 ± 0.78 mmol/l (± SD) at the beginning of the experiment to 3.08 ± 0.74 mmol/l after 20 days, and remained constant throughout the further experimental period. The extremely low initial serum cholesterol level of the calves may be due to the fact that the animals during the 24 hours between arrival and first blood sampling had only received an electrolyte solution. The inclusion of cholesterol in the diets significantly ($P < 0.01$) increased the concentration of serum cholesterol in the 0.56 and 0.93%-cholesterol groups already after 20 days, and this increase persisted during the whole experiment. For the 0.19%-cholesterol group, the increase just reached statistical significance ($P < 0.05$) at days 20, 126 and 146 of the experiment, but at the other time points, the levels of serum cholesterol in the control animals and the 0.19%-cholesterol group were not significantly different. The levels of serum cholesterol in the 0.56 and 0.93%-cholesterol groups were significantly different only at days 20 ($P < 0.01$) and 100 ($P < 0.05$) of the experiment.

It appears from table 2 that the concentrations of triglycerides and phospholipids in the serum of the calves were increased by dietary cholesterol. The level of phospholipids increased with increasing dietary choles-

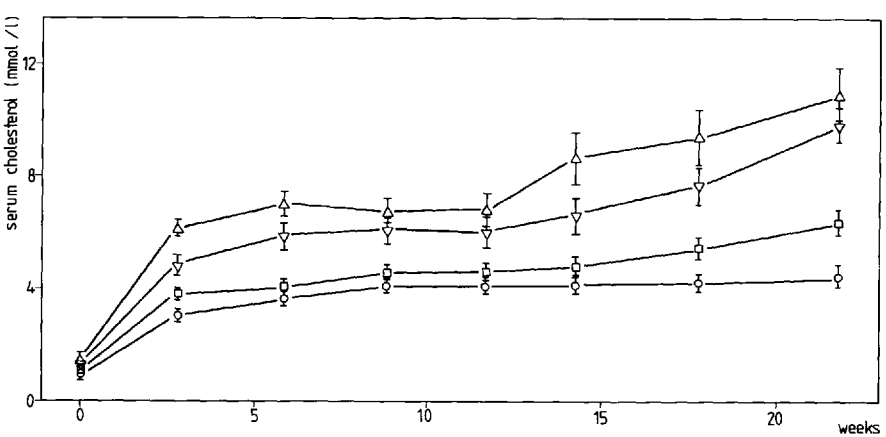

Fig. 1. Time course of the concentration of total cholesterol (means ± SE) in the serum of veal calves. Control group (n = 11), 0; 0.19%-cholesterol group (n = 12), □; 0.56%-cholesterol group (n = 12), ▽; 0.93%-cholesterol group (n = 12), △.

Table 2. Effect of dietary cholesterol on serum lipids in veal calves.

Serum lipids (mmol/l)	Cholesterol in milk replacer diet[1])			
	0 % (11)	0.19 % (12)	0.56 % (12)	0.93 % (12)
Total cholesterol	4.52 ± 0.87	6.24 ± 1.19	9.80 ± 2.45	10.92 ± 3.50
Free cholesterol	0.59 ± 0.19	1.09 ± 0.33	1.80 ± 0.60	2.14 ± 0.90
Esterified cholesterol	3.93 ± 0.69	5.15 ± 0.86	7.99 ± 1.91	8.78 ± 2.67
Ratio of free to esterified cholesterol	0.15 ± 0.02	0.21 ± 0.03	0.23 ± 0.04	0.24 ± 0.05
Triglycerides	0.14 ± 0.03	0.18 ± 0.04	0.18 ± 0.04	0.18 ± 0.03
Phospholipids	2.04 ± 0.31	2.52 ± 0.47	3.27 ± 0.57	3.48 ± 0.82

Results are expressed as means ± SD; number of animals in each group are given in parentheses. The diets were fed for 146 days. Serum lipid concentrations of the cholesterol-fed calves are significantly different ($P < 0.01$) from the values in the control calves.

[1]) Diet codes refer to the amount of added cholesterol (cf. table 1).

terol loads, whereas the level of triglycerides was already at its maximum with the lowest cholesterol supplement. In relative terms, the cholesterol-induced increase in the concentration of free cholesterol was more pronounced than that of esterified cholesterol. This is illustrated by the fact that cholesterol feeding caused a significant increment in the ratio of free to esterified cholesterol (table 2).

The lipoprotein profile of the animals at the end of the experimental period can be seen from the photograph of the pre-stained serum after

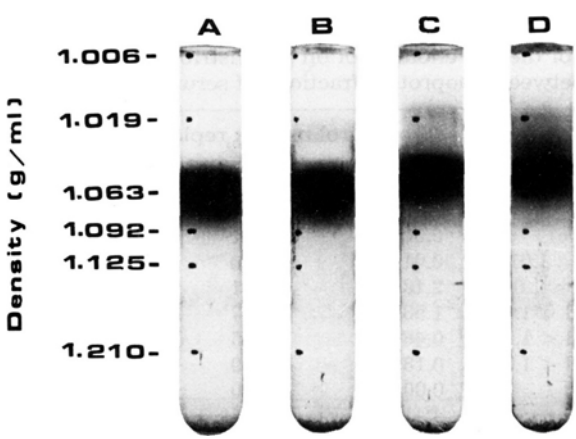

Fig. 2. Photograph of the density profile of Sudan-Black-stained lipoproteins observed after density gradient ultracentrifugation of pooled serum from calves fed milk replacers without and with added cholesterol. A, control group; B, 0.19%-cholesterol group; C, 0.56%-cholesterol group; D, 0.93%-cholesterol group. Blood was drawn at the end of the experiment.

density gradient ultracentrifugation (fig. 2). The lipoprotein profile of the calf does not resemble that of the common laboratory animals and man (19). The stained lipoprotein gradients of the control calves show VLDL at the top of the tube, a heavy band in the LDL ($1.019 < d < 1.063$) and HDL ($1.063 < d < 1.125$) region, and some residual stain at the base. Occasionally, one or two faint bands just above the heavy lipoprotein band can also be observed (5). When the calves were fed the milk replacers supplemented with cholesterol, an increase in the Sudan-Black-stained lipoproteins in the density range 1.019–1.063 g/ml were seen. This increase was essentially proportional to the cholesterol content of the diet. Furthermore, based on the intensity of the Sudan-Black staining, it would appear that dietary cholesterol induces a decrease in the density of the HDL fraction as this band moved towards the top of the tube, the covered distance being dependent on the percentage of cholesterol in the diet (fig. 2).

A more detailed insight into the changes in the individual lipoproteins can be obtained by an examination of the changes of cholesterol in the lipoprotein fractions. At the end of the experimental period, cholesterol feeding induced a marked increase in the VLDL, IDL and LDL fractions (table 3). The inclusion of 0.93 % cholesterol in the diet, when compared to 0.56 %, did not further elevate cholesterol in the IDL and LDL fractions. In contrast, the cholesterol content of the VLDL fraction was increased with 0.93 % dietary cholesterol, when compared to 0.56 % cholesterol. Cholesterol feeding caused only a slight increase in the cholesterol content of the HDL_1 fraction. Increasing concentrations of cholesterol with increasing dietary cholesterol loads were observed in the HDL_2 fraction. As to the HDL_3 fraction, 0.93 % cholesterol in the diet did not further increase the cholesterol content of this lipoprotein species, when compared with the 0.56%-cholesterol diet (table 3).

Table 3. Effect of dietary cholesterol on the distribution of cholesterol (mmol/l of whole serum) between lipoprotein fractions of serum from veal calves.

Lipoprotein fraction	Cholesterol in milk replacer diet[1])			
	0 %	0.19 %	0.56 %	0.93 %
VLDL ($d < 1.006$)	0.00	0.02	0.15	0.21
IDL ($1.006 < d < 1.019$)	0.01	0.20	1.06	1.01
LDL ($1.019 < d < 1.063$)	2.18	3.51	5.07	4.93
HDL_1 ($1.063 < d < 1.092$)	1.58	1.67	1.77	1.99
HDL_2 ($1.092 < d < 1.125$)	0.46	0.55	0.57	0.71
HDL_3 ($1.125 < d < 1.210$)	0.18	0.29	0.41	0.38
$d > 1.210$	0.00	0.00	0.02	0.01
Total	4.41	6.24	9.05	9.24
Whole serum	4.33	6.05	8.95	9.52
Recovery (%)	102	103	101	97

Blood was drawn at the end of the experimental period; lipoproteins were separated by ultracentrifugation from pool sera of each dietary group.
[1]) Diet codes refer to the amount of added cholesterol (cf. table 1).

Discussion

The present study confirms earlier reports (3–5, 11, 12, 21, 22) that the supplementation of a milk replacer diet with cholesterol results in a significant increase in the concentration of cholesterol in the serum of the young, non-ruminating bovine. In addition, we found that the effect of dietary cholesterol on the serum cholesterol level was essentially linear, at least over the range that was studied. Figure 3 illustrates this relationship for three time points during the experiment. It could be argued, however, that the upper two lines in figure 3 should in fact be drawn as S-shaped curves. This implicates that at low levels of dietary cholesterol the level of serum cholesterol is hardly affected, while at higher dietary cholesterol loads almost the same, high values of serum cholesterol are observed. Such a relationship is compatible with the observation that at most time intervals of the experiment the levels of serum cholesterol induced by the control and 0.19%-cholesterol diets and by the 0.56%-cholesterol and 0.93%-cholesterol diets, respectively, were not significantly different (fig. 1).

The serum cholesterol levels in the cholesterol-fed calves reached the new steady-state after about 3 weeks (fig. 1). It is important to note that after about 12 weeks of cholesterol feeding, the level of serum cholesterol gradually increased again. We have also seen this phenomenon in a previous study (5). After about 12 weeks of age, growth of the calves, expressed as g/day, falls, but the feed intake is still increasing (6). This means that the cholesterol intake on the basis of growth per day rapidly increases. Possibly, the amount of cholesterol otherwise incorporated in new tissues now accumulates in the serum, causing the additional increase in the concen-

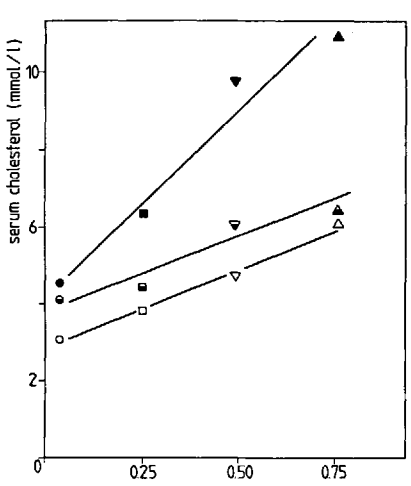

Fig. 3. Relationship between the analysed cholesterol content of the diet and the mean level of serum total cholesterol. Control group, 0; 0.19%-cholesterol group, □; 0.56%-cholesterol group, ▽; 0.93 %-cholesterol group, △. Open symbols, cholesterol levels at day 20; half-closed symbols, cholesterol levels at day 62; closed symbols, cholesterol levels at day 146.

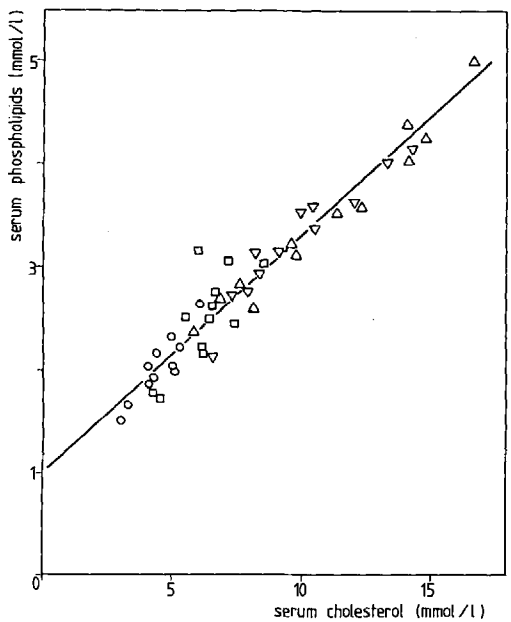

Fig. 4. Relationship between the concentrations of serum total cholesterol and serum phospholipids. For explanation of symbols, see figure 1. Blood samples were taken at day 146 of the experiment.

tration of serum cholesterol at the end of the experiment in the animals fed cholesterol. In any case, the increase cannot be due to analytical error since the cholesterol level in the control calves did not change.

Dietary cholesterol significantly increased the level of phospholipids in the serum of the calves. The increase was directly related to the amount of cholesterol in the diet (table 2). Serum triglycerides were also elevated by cholesterol in the diet, but the lowest cholesterol supplement already caused maximum concentrations. Apparently, cholesterol metabolism in calves is more closely related to phospholipid metabolism than to triglyceride metabolism. This is substantiated by the very high correlation found between serum total cholesterol and serum phospholipids (fig. 4); the correlation coefficient was 0.97 ($P < 0.001$). The linear correlation coefficient between serum cholesterol and serum triglycerides calculated for all animals, was found to be 0.35 ($P < 0.02$).

In the cholesterol-fed calves the levels in serum of both free and esterified cholesterol were increased. The ratio of free to esterified cholesterol was significantly increased in the animals fed cholesterol (table 2). In contrast, the hypercholesterolemia in cholesterol-fed rabbits is almost exclusively due to an increase in the content of serum esterified cholesterol (8, 15, 17). The cholesterol excess in the serum of cholesterol-fed calves was mainly carried in the LDL fraction. In relative terms, however, the increase was most pronounced in the VLDL and IDL fractions. Similar changes were seen in cholesterol-fed rabbits (18).

It is clear from table 3 that dietary cholesterol causes differential responses as to the cholesterol content of the various lipoprotein fractions. In the VLDL, HDL_1 and HDL_2 fractions the cholesterol concentration increased with increasing amounts of cholesterol in the diet. In contrast, the IDL, LDL and HDL_3 fractions appeared to become saturated with cholesterol when the animals were fed at least 0.56 % cholesterol. It should be emphasized, however, that these data should be interpreted with caution since upon cholesterol feeding part of the cholesterol associated with HDL_1, in the density range 1.063–1.092 mg/ml, may have shifted into the density area of LDL ($1.019 < d < 1.063$), as illustrated in figure 2.

Between individual calves there were considerable differences in the response to dietary cholesterol. At the end of the experimental period the mean serum cholesterol concentration in the 0.93%-cholesterol group was 10.92 mmol/l with individual values ranging from 5.77 to 16.65 mmol/l. Previous work with rabbits fed cholesterol established a positive correlation between the initial plasma cholesterol concentration and the increase observed after 3 weeks of cholesterol feeding (14). However, the present work with veal calves reveals a negative association between cholesterolemic response after 146 days and initial serum cholesterol level. Within the 0.93%-cholesterol group the linear correlation coefficient was found to be -0.60 ($P < 0.05$). For the other groups the correlations did not reach statistical significance, the correlation coefficient in the 0.56 % and 0.19%-cholesterol group being -0.31 and -0.03, respectively. Thus it appears that the correlation coefficient degrades with decreasing cholesterolemic response.

In conclusion, the present study has demonstrated that dietary cholesterol over the range 0–0.76 % (on the basis of air-dry matter) increased the level of serum cholesterol in veal calves either in a linear or S-shaped fashion, dependent on the time during which the animals were challenged. The dose-response relationship between dietary cholesterol and the cholesterol content of serum lipoproteins varied among the different fractions.

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References

1. Abell, L. L., B. B. Levy, B. B. Brody, F. E. Kendall: J. Biol. Chem. **195,** 357 (1952).
2. Bartlett, G. R.: J. Biol. Chem. **234,** 466 (1959).
3. Beynen, A. C., L. G. M. van Gils: Z. Ernährungswiss. (in press).
4. Beynen, A. C., L. G. M. van Gils: Amer. J. Clin. Nutr. **37,** 155 (1983).
5. Beynen, A. C., L. G. M. van Gils: Nutr. Rep. Int. (in press).
6. Beynen, A. C., L. G. M. van Gils: Z. Tierphysiol., Tierernährg. u. Futtermittelkde. **49,** 49 (1983).
7. Böttcher, C. J. F., C. M. van Gent, C. Pries: Anal. Chim. Acta **24,** 203 (1961).
8. Camejo, G., V. Bosch, C. Arreaza, H. C. Mendez: J. Lipid Res. **14,** 61 (1973).
9. Eggstein, M.: Klin. Wschr. **44,** 267 (1966).

10. Folch, J., M. Lees, G. H. Sloane Stanley: J. Biol. Chem. **226,** 497 (1957).
11. Jacobson, N. L., M. Richard, P. J. Berger: J. Nutr. **103,** 1533 (1973).
12. Jacobson, N. L., M. Richard, P. J. Berger, J. P. Kluge: J. Nutr. **104,** 573 (1974).
13. Nordby, H. E., S. Nagy: J. Chromatogr. **75,** 187 (1973).
14. Roberts, D. C. K., C. E. West, T. G. Redgrave, J. B. Smith: Atherosclerosis **19,** 369 (1974).
15. Rodriguez, J. L., G. C. Ghiselli, D. Torreggiani, C. R. Sirtori: Atherosclerosis **23,** 73 (1976).
16. Röschlau, P., E. Bernt, W. Gruber: Z. klin. Chem. klin. Biochem. **12,** 403 (1974).
17. Ross, A. C., D. B. Zilversmit: J. Lipid. Res. **18,** 169 (1977).
18. Scholz, K. E., A. C. Beynen, C. E. West: Atherosclerosis **44,** 85 (1982).
19. Terpstra, A. H. M., F. J. Sanchez-Muniz, C. E. West, C. J. H. Woodward: Comp. Biochem. Physiol. **71 B,** 669 (1982).
20. Terpstra, A. H. M., C. J. H. Woodward, F. J. Sanchez-Muniz: Anal. Biochem. **110,** 149 (1981).
21. Wiggers, K. D., N. L. Jacobson, R. Getty: Atherosclerosis **14,** 379 (1971).
22. Wiggers, K. D., N. L. Jacobson, R. Getty, M. Richard: Atherosclerosis **17,** (1973).

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