Enzymatic Degradation of Egg Yolk Cholesterol

A thesis submitted in total fulfilment of the requirements of the degree of Master of Science

by

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March 1998

ACKNOWLEDGEMENTS

I want to sincerely thank my mother and father for their wonderful encouragement and support, both intellectual and financial, throughout the course of my life, and to gratefully acknowledge the opportunity they have given me to reach a higher level of education.

Special thanks is extended to my supervisors Dr Tran Viet Hung and Mr Robert Black of Food Science Australia and Assoc. Prof. Maurie Trewhella of University of Technology for their invaluable advice, encouragement and expert constructive criticism throughout this work. Particular thanks to Dr Tran Viet Hung and Assoc. Prof. Maurie Trewhella for their patience and persistence.

I would also like to thank LiHui Lui and Milica Rodavanovic, fellow postgraduate students for sharing the experience of postgraduate studies with me, and Gail Di Gregorio of Food Science Australia, for her support and good humour during the sometimes frustrating first year of my thesis. Thanks also to Hein Hamerjylink for providing a much-needed diversion to my studies.

Thanks to Drs Heather Craven and John Coventry and Ms Vivien Boghossian of Food Science Australia, for expert technical advice, and Daphne Wolmersley and Lorraine Ruddick for their helpful assistance.

Many thanks to Mr Kym Butler of the Victorian Institute of Animal Science, for assistance with the design of experiments and statistical analysis, Mr Vince Murone (trouble shooter extraordinaire) of Victoria University of Technology, for assistance with GC-MSD analysis and Dr Harry Laan, post-doctoral fellow from the University of Groningen in the Netherlands, for valuable discussion and to Stella Halkiadakis, Jill Vince, Ian Sullivan and Min Nguyen of the School of Life Science Sector, Victoria University of Technology for their helpful assistance with equipment.

Finally to all my family and friends, whose insistence on running commentaries as to the progress of my thesis at every opportunity secured it's completion, I extend sincere thanks.

This work was made possible through facilities provided by Food Science Australia and Victoria University of technology and the financial support provided by a grant from the Egg Industry Research Council and a research scholarship from Victoria University of Technology.

DECLARATION

The studies described in this thesis have not been previously submitted for any other degree at this or any other University. The studies presented in this thesis represent my own original work except where due acknowledgment has been made. This thesis is less than 100,000 words in length, exclusive of tables, bibliographies, appendices and footnotes.

Speroulla Christodoulou

For Mum, Dad and my grandparents, Γιαγια and Παπον

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ABSTRACT

A rapid, reliable and accurate gas chromatographic method for the determination of cholesterol in egg yolk and processed foods was developed from a method of Kovacs (1990). A sample is heated in a sealed tube with a solution of potassium hydroxide in ethanol containing the internal standard, dihydrocholesterol. The sterols are then isolated by solvent extraction, converted to trimethylsilyl (TMS) derivatives and subjected to gas chromatography.

The method was used to determine the cholesterol content of a number of foods in order to demonstrate its application to more complex matrixes. Among egg-based products, Italian egg spaghetti had a lower cholesterol content (0.14-0.74mg/g) than egg noodles (0.87-1.33mg/g). Some egg rolls contained cholesterol levels as high as 1.72-3.61mg/g. Among meat-based foods, ham products had cholesterol levels similar to pork cakes or pork loafs (0.59-0.67mg/g and 0.66-0.81mg/g respectively). Fish balls and cheddar cheese contained almost the same level of cholesterol (0.93mg/g and 0.95mg/g, respectively). Of all foods investigated, chicken liver, chicken paste and prawn contained the highest levels of cholesterol.

The capacity of four cholesterol oxidases, from *Pseudomonas fluorescens* (Pf), *Nocardia erythropolis* (Ne), *Brevibacterium species* (Bs) and *Streptomyces species* (Ss), and cholesterol reductase isolated from cucumber leaves to degrade cholesterol in egg yolk was investigated.

Incubation of egg yolk with a cholesterol reductase for 72h at 37°C resulted in minimal (3.6%) cholesterol reduction. In contrast, up to 93.4% of the total available cholesterol was degraded by cholesterol oxidases from *Pseudomonas fluorescens* and *Streptomyces species* under similar conditions. At 4°C, cholesterol oxidase from *Pseudomonas fluorescens* could degrade up to 64.9% cholesterol

after 48h; cholesterol lowering was barely evident at 4°C with the other cholesterol oxidases. The most effective temperature was 37°C for Bs cholesterol oxidase, and 45°C for the other cholesterol oxidases. The enzyme derived from *Pseudomonas fluorescens* had an effective temperature range from 15 to 60°C, and from 5 to 37°C it was clearly superior to the other three oxidases. The capacity of the cholesterol oxidases to degrade cholesterol in egg yolk followed the sequence Pf > Ne > Ss > Bs. The optimal enzyme concentration for cholesterol degradation was the lowest for Pf cholesterol oxidase at 0.5U/μmole cholesterol. Even at the low concentration of 0.125U/μmole cholesterol, Pf cholesterol oxidase degraded up to a third of the egg cholesterol in 2h.

The cholesterol degrading activities of *Rhodococcus equi*, *Rhodococcus erythropolis* and two other unspeciated isolates of *Rhodococcus* were investigated in egg yolk and milk. These microorganisms, when grown on a cholesterol containing medium, resulted in halo formation around colonies of the *Rhodococcus species* consistent with the production of cholesterol degrading enzymes. The ability of *Rhodococcus equi* No. 23 to degrade cholesterol in whole homogenised UHT milk or preparations diluted to half strength was minimal. However, *R. equi* No. 23 degraded up to 52.7% of cholesterol in a 1/8 strength preparation of UHT milk. In these preparations cholesterol degradation was further enhanced by growth of *R. equi* No. 23 cells used as the inoculum in UHT milk rather than in an artificial medium, prior to determination of cholesterol degradation.

The cholesterol degrading system of *R. equi* No. 23 was equally effective in reducing the concentrations of cholesterol in egg yolk. The rate of cholesterol degradation was also enhanced by pre-incubations of *R. equi* No. 23 cells in yolk compared with artificial medium. Cholesterol degradation in egg yolk was almost complete after growth of *R. equi* No. 23 for 7d at 37°C.

PUBLICATIONS

The following articles have been published or submitted for publication during the course of this thesis:

Christodoulou S., T. V. Hung, M.A. Trewhella and R. Black (1994) *Enzymatic degradation of egg yolk cholesterol* **J. Food Protection.** 57(10), 908-912.

Christodoulou S., T. V. Hung, J. Coventry and M.A. Trewhella *Microbial degradation of egg yolk cholesterol* **Aust. J. Dairy Technol.** (submitted).

COMMUNICATIONS

The following communications have been presented to learned societies:

Christodoulou S., T. V. Hung, M.A. Trewhella and R. Black (1993) *Enzymatic degradation of egg cholesterol* **26th Convention Proceedings AIFST** 2nd-6th May, Adelaide PP.4.

Christodoulou S., T. V. Hung, M. A. Trewhella and R. Black (1993) *Cholesterol content of some western foods and oriental analogues* **X V International Congress of Nutrition** 26 September-1st October, Adelaide P31.

Christodoulou S., T. V.Hung, M. A. Trewhella and R. Black (1994) *Degradation of milk and egg cholesterol by enzymes and microorganisms* **24th International Dairy Congress** 18th-22nd September, Melbourne.

LIST OF ABBREVIATIONS

ABS	Australian Bureau of Statistics
Bs	Brevibacterium species
BSTFA	N-O-bis-(trimethylsilyl)-trifluoroacetamide
CHD	coronary heart disease
COD	cholesterol oxidase
CPIB	p-chlorophenoxyisobutyrate
CR	cholesterol reductase
CVD	cardio vascular disease
DEAE	2-(diethyl-amino)ethyl-Sephadex
E	enzyme
EI	enzyme-intermediate complex
ES	enzyme-substrate complex
FAD	flavin adenine dinucleotide
FDA	Food and Drug Administration
FID	flame ionisation detector
GC	gas chromatograph
GC-MS	gas chromatography-mass spectrometry
HDL	high density lipoprotein
HMG-CoA	human gonadotrophin CoA
HPLC	high performance liquid chromatography
JCM	Japan Collection of Microorganisms
LDL	low density lipoprotein
LRC-CPPT	Lipid Research-Coronary Primary Prevention Trial
LSD	least significant difference
MSD	mass selective difference
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
NADH	nicotinamide adenine dinucleotide

NADPH	nicotinamide adenine dinucleotide phosphate
NCEP	National Cholesterol Education Program
Ne	Nocardia erythropolis
NHFA	National Heart Foundation
NHMRC	National Health and Medical Research Council
P	product
Pf	Pseudomonas fluorescens
PVPP	polyvinyl polypyrrolidone
s.e.	standard error
S/U	saturated/unsaturated
SFE	supercritical fluid extraction
Ss	Streptomyces species
TLC	thin-layer chromatography
TMS	trimethyl silyl
U/mmole	units of enzyme per micromole
UHT	ultra high temperature

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CHAPTER 1

Literature Review

1.1 Egg Nutritional Value

Eggs are among the most nutritious foods known, providing a unique, well-balanced source of nutrients for persons of all ages. They contribute to the body's nutrient needs during rapid growth, and are an excellent food for young children and teenagers. Their high nutrient content, comparatively low caloric value and ease of digestibility make eggs valuable in many therapeutic diets for adults (Cook and Briggs, 1977).

Although eggs contain about 74% water, they are a rich source of high quality proteins. Experimental nutritionists use egg proteins as a standard for measuring the quality of other food proteins (Cook and Briggs, 1977). Eggs are also an important source of iron, phospholipids, trace minerals, vitamin A, B (including B_{12}), E and K. As a source of vitamin D, eggs rank second only to fish-liver oils. Eggs are low in calcium, however, and contain very little, or no, vitamin C (Cook and Briggs, 1977).

1.2 Egg Composition

Whole egg is composed of approximately 64% white and 36% yolk (Pratt, 1975). The yolk contains all of the egg lipid; Table 1.1 gives the composition of egg yolk and lists the components of the yolk lipid.

Table 1.1 Egg yolk composition (Pratt, 1975).

Component	g/100g fresh egg yolk
water	51.1
protein	16.0
ash	1.7
carbohydrate	0.6
lipid	30.6
triglyceride	20.0
phospholipid	8.7
cholesterol	1.6
cholesteryl ester	trace

The fatty acids of the phospholipid are predominantly unsaturated; the main unsaturated fatty acids are oleic acid (45.5±1.7%) and linoleic acid (13.1±2.0%) (O'Dea and Sinclair, 1991).

Egg yolk has been described as a concentrated source of cholesterol (Gurr *et al.*, 1989). Reported measurements of egg yolk cholesterol have ranged from 195-270mg/egg (Feeley, 1972; Beyer and Jensen, 1989a; Holden *et al.*, 1989,).

Most of the cholesterol in egg yolk is found in the low density lipoprotein (LDL) (Gornall and Kuksis, 1971). The composition of LDL in egg yolk comprises of protein (11-17%), phospholipid (22-28%) and neutral lipid (59-67%) (Holdsworth and Finean, 1972).

Egg yolk LDL particles appear to be spheres of lipid surrounded by a layer of glycoprotein, phospholipid and cholesterol with hydrophobic groups on the inside

and hydrophilic groups oriented toward the outside (Aihara *et al.*, 1988a). Electron microscopy and X-ray diffraction strongly support the concept of a mosaic structure of the surface of the LDL particles in which protein and lipid cover separated areas of the surface and do not form strong associations with each other (Holdsworth and Finean, 1972).

1.3 Cholesterol Synthesis and Utilisation

Cholesterol in man is synthesised in many tissues, especially the liver and the small bowel, and is also obtained from the diet (McGilvery, 1979). It is an essential substance in the body, a vital constituent of all cell walls, and is also the precursor of faecal steroids, bile acids and steroid hormones in animals (Lehninger, 1975b). The origin and destination of contributions to the cholesterol balance in the body is shown in Figure 1.1.

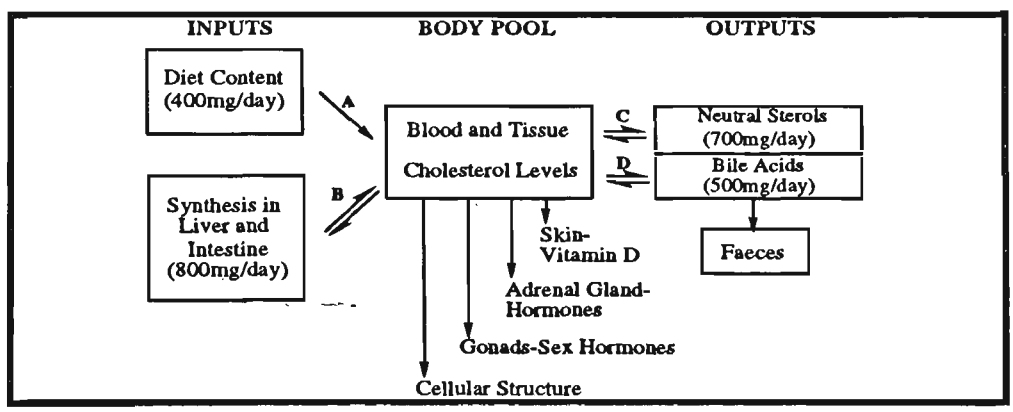


Figure 1.1 The inputs and outputs that contribute to cholesterol balance. Liver and intestinal synthesis in the body normally contribute about twice as much cholesterol to the body pool as does the normal human diet, 800 versus 400mg per day. Cholesterol is converted in the liver to neutral sterols and bile acids that are released to the intestine and recycled or excreted from the body. Loss in the faeces is usually compensated for by the inputs from diet and synthesis. Changes in dietary cholesterol input would be expected to be compensated for by changes in synthesis to maintain the balance under normal conditions (Naber, 1991).

Most of the cholesterol synthesised or absorbed from the diet each day is used to replace the bile acids and cholesterol lost in the faeces (McGilvery, 1979). If all systems are functioning optimally the body can adjust cholesterol synthesis and excretion, reducing or increasing each as appropriate to balance cholesterol intake in the diet (O'Dea and Sinclair, 1991). Thus, concentrations of cholesterol can be maintained near a constant level day-to-day, and perhaps month-to-month, despite variations in demand and in dietary supply (McGilvery, 1979).

This physiological feedback system operates in the majority of the adult population, however, in 20-30% this system does not function (Davidson, 1990). Several human studies have shown that people can be classified into two categories: those with increased plasma cholesterol levels and those with unchanged cholesterol levels, following cholesterol intake (Kestin *et al.*, 1989; Simons *et al.*, 1988).

Transport of Cholesterol in the Bloodstream

Atherosclerosis is a disease caused by an accumulation of cholesterol in the arteries in the form of bulky plaques that inhibit blood flow and eventually cause a heart attack or stroke (Soehnlen, 1987). LDL is the major cholesterol-carrying lipoprotein in the blood and high levels of LDL can result in the development of atherosclerosis (Soehnlen, 1987). The level of LDL in the blood is affected by specialised proteins called LDL receptors. These receptors bind the LDL particles, which are then taken into the cells where they are broken down, and the cholesterol, which is needed by the cell, is utilised (Brown and Goldstein, 1984).

Cholesterol is also transported by another lipoprotein, high density lipoprotein (HDL), which has the opposite type of association with atherosclerosis (Nestel *et al.*, 1989). Elevated levels of HDL-cholesterol, in particular HDL_2 particles, are associated with reduced risk of coronary heart disease (Mattson and Grundy, 1985). A significant rise in the HDL_2 distribution was reported in men fed two eggs daily, against background diets either high or low in saturated fat, although mean HDL did not change. This may represent a means of clearing dietary cholesterol (Kestin *et al.*, 1989).

The levels of LDL and HDL appear to influence the deposition and accumulation of cholesterol in artery walls, a critical factor in the etiology of atherosclerosis and occlusive vascular disease (Nestel *et al.*, 1989).

An hereditary condition called hypercholesterolaemia causes elevated LDL concentrations. This is as a result of either, an absence or deficiency of the LDL-receptor site protein in plasma membranes, or a failure in the mechanism for endocytosis of the attached LDL from the receptor site (McGilvery, 1979). This disease causes high blood cholesterol and heart attacks in persons under 60 years of age (Soehnlen, 1987) and a high dietary intake of cholesterol could be a contributing factor (Brown and Goldstein, 1984). Therefore, people with a family history of heart attacks or strokes, those particularly susceptible to damaging effects of LDL, should further restrict intakes of dietary cholesterol (Brown and Goldstein, 1984).

1.4 Epidemiological Studies and Clinical Trials

Many different types of epidemiological studies have quantitatively defined the relationship between cholesterol levels and coronary heart disease (CHD) risk (Rifkind, 1985). Studies such as: Framingham, an investigation that helped elucidate the precursors of coronary heart disease (Corday and Corday, 1975); and Lipid Research Clinics-Coronary Primary Prevention Trial (LRC-CPPT), a double-blinded, primary prevention study of the effect of cholestyramine on men who had hypercholesterolaemia (Gotto and Phil, 1984), have been especially important in establishing the fact that the higher the blood cholesterol, the greater the risk of CHD (Levy, 1985; Rifkind, 1986). The LRC-CPPT findings provided evidence that reduced incidence of CHD is mediated chiefly by total plasma cholesterol and LDL levels (Anon., 1984).

Clinical trial findings are in good agreement with what was suggested by the epidemiological studies, namely, that each 1% reduction in plasma cholesterol is associated with an approximately 2% reduction in CHD manifestations (Rifkind, 1986).

A characteristic of many of these trials was the selection of middle-aged subjects suffering from hypercholesterolaemia. Dietary recommendations based on extrapolations made to the rest of the population with less severe elevations of cholesterol, have consequently been regarded by some as unjustified on present evidence (Kronmal, 1985; Ahrens, 1985; Levy, 1985). Nonetheless, these trials have provided some evidence that lowering dietary lipids is beneficial in reducing

the risk of CHD, and that there exists a possible dose (cholesterol-lowering) response relationship (Rifkind, 1986).

1.5 Dietary Studies

Several dietary studies (O'Dea *et al.*, 1991; Kestin *et al*, 1989b) highlight a possible correlation between cholesterol levels found in human plasma and background diet. Evidence suggests that it is primarily saturated fat of animal origin (fat in dairy and meat products) that raises cholesterol levels. Polyunsaturated and monounsaturated fats/oils have been shown to have quite different effects on the lipoprotein profile (O'Dea and Sinclair, 1991).

Low-fat diets containing two eggs per day when supplemented with either butter or olive oil resulted in the rise and fall of total plasma cholesterol, respectively. This effect was related to the changes in LDL-cholesterol and HDL-cholesterol levels. Rises in total plasma cholesterol concentration in response to the addition of butter was entirely due to rises in LDL-cholesterol, however, HDL-cholesterol did not change (O'Dea and Sinclair, 1989).

Kestin *et al.* (1989a) observed no significant change in mean total LDL-cholesterol or HDL-cholesterol plasma concentrations in response to dietary cholesterol when changing from a high fat to a low-fat diet. However, considerable changes did occur in the average size of HDL particles. Proportionately more was present as HDL_2, reflecting a compensating mechanism for excess dietary cholesterol through the scavenging potential of HDL (Kestin *et al.*, 1989a).

A significant rise in serum cholesterol (10%) was reported when changing from two, to as many as six eggs per week, under well controlled experimental conditions (Beynen and Katan, 1983). In comparison, a rise in serum cholesterol of less than 10% was measured for a control diet of low cholesterol content (Kestin *et al.*, 1989a). Yet others found no significant elevations in plasma cholesterol concentrations in men ingesting cholesterol equivalent to 2 eggs per week against two background diets (high saturated fat vs fat modified) (Kestin *et al.*, 1989a), suggesting that the influence of dietary cholesterol may be independent of the type of fatty acids present in the dietary fat.

One reason for the discrepancies is the now well established variability in human response to dietary cholesterol (Beynen and Katan, 1985; Simons *et al.*, 1988; Kestin *et al.*, 1989a).

Hyper- and Hypo- Responders

Hypercholesterolaemic people can be further divided into two groups: hyper-responders and hypo-responders or dietary-sensitive and dietary-insensitive individuals, respectively. Dietary sensitive individuals were found to be more responsive to a background diet low in fat than dietary insensitive individuals when supplemented with cholesterol (Nestel *et al.*, 1989). Hypercholesterolaemic individuals who are sensitive to dietary fat reduction have shown larger responses to dietary cholesterol than either fat-insensitive hypercholesterolaemic subjects or normocholesterolaemic individuals (Kestin *et al.*, 1989a).

A 3.7% rise in plasma cholesterol, a 4.9% rise in LDL-cholesterol and a 5.4% rise in HDL-cholesterol was seen in hypercholesterolaemic men and women given approximately 700mg a day of yolk cholesterol in a double-blinded, crossover study while on a background diet containing 25% of energy as fat, of which 9% was derived from saturated fat. Normocholesterolaemic individuals experienced small, non-significant rises in total LDL-cholesterol and HDL-cholesterol (Kestin *et al.*, 1989a).

O'Dea and Sinclair (1991) indicated that in normocholesterolaemic subjects the consumption of one egg a day can lower plasma cholesterol, provided the background diet is low in saturated fat (low fat diet or diets containing mono- or polyunsaturated fat). However, these beneficial affects were completely negated when butter was added at a relatively low level (10% energy). This highlights the important interaction of dietary cholesterol and saturated fat, indicating clearly that if eggs are to be consumed regularly then the saturated fat content of the diet should be kept to a minimum (O'Dea and Sinclair, 1989). Therefore, those suffering from hypercholesterolaemia should use egg yolk in moderation, and normocholesterolaemic subjects need not restrict dietary cholesterol unduly (Kestin *et al.*, 1989a).

1.6 Association between Cholesterol, Cardiovascular and Coronary Heart Disease

Coronary heart disease (CHD) and cardiovascular disease (CVD) are leading causes of death for all ages in Australia and North America. Coronary heart disease accounted for half of all deaths in Australia and is responsible for a

significant proportion of premature deaths in adults (NHFA, 1990). In America, CVD is responsible for more than 550,0000 deaths in the US each year, more deaths than all forms of cancer combined (Consensus conference, 1985). The sense of urgency in research emphasis to isolate the possible causes of CVD and to find a cure has led to a spread of misinterpretations among the public, and even among the scientific community, and has given rise to a controversial issue (Soehnlen, 1987).

Implications made by several epidemiological studies such as the Framingham study (Kannel and Gordon, 1974), the Coronary Primary Prevention Trial (Gordon *et al.*, 1986), the Multiple Risk Factor Intervention trial (Rifkind, 1986) and the Lipid Hypothesis (Soehnlen, 1987), have led health authorities to make dietary recommendations increasing public awareness of the correlations between dietary cholesterol, blood cholesterol and CVD. In fact, these investigations failed to confirm any significant effect of dietary cholesterol on serum cholesterol by either feeding cholesterol (Kestin *et al.*, 1989a) or eggs (Flynn, 1979; Hegsted, 1986; Sidhu, 1989; O'Dea and Sinclair, 1989 and 1991).

1.6.1 Health Authority Response

Since epidemiological studies have shown evidence of cholesterol-lowering by cholesterol-lowering drugs or by fat-modified diets, Australians are encouraged to reduce total fat intake, mainly saturated fat, because of its effect on plasma cholesterol. A decline of CHD mortality has been attributed to the increased consumption of polyunsaturated fats in the Australian diet. The energy from

saturated fatty acids omitted from people's diets has been replaced by complex carbohydrates, monounsaturated and polyunsaturated fats (NHMRC, 1992).

Similarly, in America, the National Cholesterol Education Program (NCEP) has recommended that dietary cholesterol, total fat and saturated fat be limited, both for those at increased risk of CHD and for the general public (Expert Panel, 1988). Based on these factors, the NCEP, American Heart Association and Surgeon General recommend reducing dietary cholesterol to about 300mg/day (Fairchild, 1991).

A typical Australian consumes about 450mg of cholesterol per day, about 185mg of this coming from eggs and dairy fat (Sidhu, 1989). The National Heart Foundation of Australia has provided guidelines recommending the intake of cholesterol-rich foods be reduced and no more than two eggs should be consumed per week due to their cholesterol content (NHFA, 1989).

Therefore, efforts to control serum cholesterol by dietary changes are not limited to the cholesterol in the diet; dietary fatty acid composition has a powerful effect on the cholesterol level in the serum (Keys, 1984). General dietary recommendations outlined by an NHMRC Working Party (NHMRC, 1992) for Australians included reducing the energy intake from saturated fat from 15% to 10% of energy. Reducing saturated fatty acids to about 9% could produce an average reduction of 23mg/dL in serum cholesterol (Keys, 1984).

1.6.2 Public Response

In America, the proportion of people believing that a reduction of blood cholesterol would have a large effect on heart disease increased following the release of the results from the Coronary Primary Prevention Trial (72% of those surveyed, compared with 64%; Gordon *et al.*, 1986). Dietary changes compared with cholesterol-lowering drugs, were most frequently chosen as ways to control the blood cholesterol level. For instance, reducing dietary fat was believed to be as important as reducing dietary cholesterol. By 1986, 23% of adults reported that they made dietary changes specifically to lower their blood cholesterol level compared with 14% in 1983 (Schucker *et al.*, 1987a). In a 1996 study by National Family Opinion conducted by the American Egg Board, a consumer concern about cholesterol in the foods they ate further declined to 3.5%, the lowest since 1986, with 50% of those questioned citing fats in the diet as the chief culprit of high cholesterol (Anon, 1996).

Before 1983, physicians attributed less preventative value to lowering blood cholesterol than the public, indicative of a reluctance on the part of physicians to make strong endorsements in the absence of conclusive scientific evidence. However, between 1983 and 1986, with the publication of the LRC-CPPT, physicians were more convinced of the benefit of lowering high blood cholesterol levels, and were treating patients accordingly (Schucker *et al.*, 1987b). Elevated blood cholesterol levels were regarded with a level of seriousness approaching that accorded high blood pressure or the risk from smoking, by both practising physicians and the public (Schucker *et al.*, 1987b).

Egg Consumption

Dietary recommendations by prominent health authorities based on limited, controversial understanding, coupled with the high level of cholesterol in egg yolk were a likely contributing factor to the decrease in egg consumption experienced by the egg industry. In Australia, the apparent per capita annual consumption of eggs has decreased from 140 to 126 since 1985 (ABS, 1993) and in the U.S. from 303 to 235 during the past twenty years (Elkin *et al.*, 1993).

However, the consumption of processed egg products has continued to increase due to the excellent consumer demand for pre-cooked and convenience products. Perhaps one of the best avenues for increasing per capita consumption of egg is through new processing and product concepts (Larsen and Froning, 1981).

1.6.3 Industry Response

Motivated by public concern over cholesterol, the food industry in Australia recognised the public demand for low-cholesterol foods. The Meat and Livestock Corporation market leaner cuts of meat, and the Dairy Corporation now produce low-fat products (Catalyst, 1991).

Like other food organisations, the Egg Industry Research and Development Councils have defined research priorities devoted to the development of technologies for the removal of cholesterol from eggs and egg-based products.

A market potential of cholesterol-reduced foods including egg-based products is estimated at 25 billion dollars (Hegenbart, 1989). Any reduction in the cholesterol content of market eggs would be valued by both consumers and egg producers (Beyer and Jensen, 1992).

1.7 Low Cholesterol Egg Products-Problem and Approach

1.7.1 Selective Breeding

Plasma cholesterol levels may represent episodic synthesis and excretion of cholesterol by the liver linked to feed intake or the ovulation period of the hen (Beyer and Jensen, 1992).

The major excretory route for cholesterol is provided by the egg (Sutton *et. al.*, 1984, Sim *et al.*, 1980). Egg yolk cholesterol is important for the developing embryo, and is transferred to a large extent from the yolk to the embryo in the final stages of incubation (Weiss and Scott, 1979). Increase in excretion of cholesterol via the egg also enables the hen to prevent hypercholesterolaemia when ingesting large levels of dietary cholesterol (Sutton *et al.*, 1984).

The hen also needs to synthesise cholesterol for use as structural components of cell membranes, as a precursor to sex and adrenal hormones, vitamin D and the bile acids (Hargis, 1988).

Attempts to produce a low cholesterol egg by selective breeding and dietary modification, have been based on the theory that plasma cholesterol levels in the

hen have a direct influence on those found in the yolk. However, these attempts have yielded only moderate results, adding credence to the alternative theory that the concentration of plasma cholesterol in the hen is not closely associated with that found in the egg (Weiss *et al.*, 1967b, Washburn and Nix, 1974b, Sutton *et al.*, 1984).

Eggs collected from nineteen producers varied in cholesterol content, according to egg weight, yolk weight, hen age, egg production and dietary protein (Beyer and Jensen, 1989a). Eggs from broiler breeders contain higher concentrations of cholesterol than eggs from commercial layer strains (Turk and Barnett, 1971) and moulted flocks have a higher level of total cholesterol than non-moulted flocks due to larger egg size (Beyer and Jensen, 1989a). These studies determining the effects of various parameters on egg cholesterol levels confirm the possibility of genetically selecting for decreased egg yolk cholesterol (Bair and Marion, 1978).

Eggs from seven inbred lines of chickens showed significant differences in yolk cholesterol (Bair and Marion, 1978). Other experiments indicate that strain of commercial layer is not a factor affecting cholesterol levels (Beyer and Jensen, 1989a), as (1) no significant differences in total egg cholesterol or cholesterol concentrations in eggs have been found between commercial egg layer strains (Turk and Barnett, 1971), and (2) significant differences in yolk cholesterol have been seen in the same strain at two different ages (Bair and Marion, 1978).

These findings agree with those of Turk and Barnett (1971) who found considerable variation among individual eggs from the same flock in the concentration of cholesterol as well as in the total amount of cholesterol per egg

regardless of the age, feed, management or strain. They concluded that there were not enough differences in the amount of cholesterol in the eggs laid by birds of several commercial strains to provide a basis for choosing one strain over another.

These variations, even among birds of the same strain, indicate the difficulties for breeders to select for, and maintain, low cholesterol egg yolk concentration. Many of these variables become interrelated with the passage of time, and this may cause difficulties for breeders when trying to maintain low cholesterol egg yolk concentration in selected strains.

Genetic differences between different strains of chickens may possibly result in differences in cholesterol metabolism (Kondra and Hodgson, 1961; Washburn *et al.*, 1975). However, birds chosen for high and low oxygen-consumption, which theoretically alters metabolic rate, exhibited altered rates of cholesterol and lipid synthesis, but genotype did not appear to alter the minimum level of cholesterol maintained in the egg (Sutton *et al.*, 1984). Genetic selection to develop a chicken line which could produce low cholesterol eggs has only yielded moderate results (2.4% reduction) (Hargis, 1988; Naber and Biggert, 1985).

Species Variation in Egg Yolk Cholesterol Concentration

Variation has been found among various species of birds in the concentration of cholesterol as well as in the total amount of cholesterol per egg. Significant differences were found between domestic and wild genetic groups for turkeys and ducks (Bair and Marion, 1978). Eggs from turkey, duck and quail contain higher concentrations of cholesterol than chicken eggs (Turk and Barnett, 1971),

therefore a shift in consumption from chicken to turkey, duck or quail groups would not reduce dietary cholesterol.

In conclusion, cholesterol levels in the hen result from a balance of dietary intake, biosynthesis, and excretion of cholesterol and cholesterol by-products (Sutton *et al.*, 1984) with yolk cholesterol concentrations for a given hen remain relatively constant (Bartov and Reiser, 1973). Even the age effect whereby total egg cholesterol increased as birds aged did not alter the concentration of yolk cholesterol, the observed effect was a result of increased egg size (Turk and Barnett, 1971).

The moderate results obtained through genetic selection (Hargis, 1988; Naber *et al.* 1976; Marks and Washburn, 1977), indicate that there may be a low heritability for low-cholesterol concentration. Selection for low cholesterol levels has usually only been effective in the upper cholesterol range (Marks and Washburn, 1977), again suggesting the importance of a basal level of cholesterol required for egg formation (Sutton, 1984).

The increased efforts required to decrease yolk cholesterol by this approach would undoubtedly be expensive (Bair and Marion, 1978). A more economical approach for producing low cholesterol eggs could be through modification of the hen's diet.

1.7.2 Diet Modification

The cholesterol content of egg yolk has been found to be affected by a number of factors including dietary supplements (Washburn and Nix, 1974b). The amount of cholesterol biosynthesis by the liver (Weiss *et al.*, 1967a) has been shown to vary significantly with dietary treatment (Vargus *et al.*, 1986). Some of the dietary factors identified as influencing yolk cholesterol are the addition of the plant sterols (β-sitosterol and soy sterol), the quantity and type of fatty acids present and the type and quantity of fibre present (McDonald and Shafey, 1989).

Plant Sterols The inclusion of sterols, other than cholesterol in the diet has been shown to reduce yolk cholesterol (McDonald and Shafey, 1989).

Plant sterol supplemented diets had no effect on egg cholesterol levels, but diets supplemented with corn oil, or corn oil and plant sterols, showed small, but significant increases in the level of egg cholesterol. Chickens bred from hens fed a commercial low-fat laying diet supplemented with 4% plant sterols, with or without 10% corn oil, had significantly reduced levels of egg cholesterol (up to 50%) (Konlande and Fisher, 1969; Kudchodkar, 1976). This was thought to be related to cholesterol absorption (Kudchodkar, 1976), the ability to absorb dietary cholesterol being highly dependent upon the nature of dietary oil (Sim and Bragg, 1978; Sim *et al.*, 1980).

Sitosterol Standard rations supplemented with 2% or 4% sitosterol emulsion reduced egg cholesterol by as much as 35%. Maximal effects were obtained at high concentrations of sitosterol fed for longer periods (Clarenburg *et al.*, 1971).

Sitosterol acts by interfering with the intestinal absorption of dietary enterohepatically circulating cholesterol, thus, lowering cholesterol levels by promoting faecal excretion of sterols and their degradation products. This suggests that the inclusion of adequate amounts of sitosterol in an absorbable form in the standard diet of laying hens would profoundly affect the egg sterol content (Clarenburg *et al.*, 1971). It has been postulated that the presence of sitosterol in egg yolk (3.8%) may also depress intestinal absorption of the reduced egg cholesterol content by the human consumer (Clarenburg *et al.*, 1971).

A conflicting report by Kudchodkar *et al.* (1976), in which hens were fed a commercial low-fat diet supplemented with β-sitosterol, provided little evidence that sitosterol passed through the intestinal barrier. Up to 93.4% of fed β-sitosterol was recovered in the faeces, with up to 80% of fed cholesterol being absorbed. A similar effect was seen when hens were fed the same diet with the inclusion of 10% corn oil and 4% plant sterols. The effect of feeding sitosterol on egg cholesterol levels was insignificant and unless hens were fed high β-sitosterol diets there was no evidence of β-sitosterol in the eggs (Kudchodkar *et al.*, 1976). Kudchodkar *et al.*, (1976) reported that 2.2% of the absorbed dose of β-sitosterol was deposited in eggs laid by hens fed high β-sitosterol diets, compared to the 3.8% observed by Clarenburg *et al.* (1971).

Soy Sterols Sim and coworkers reported that reduction of yolk cholesterol levels between 16 and 33% resulted when hens were fed soy sterols combined with various saturated and unsaturated oils (Sim and Bragg, 1978; Sim *et al*, 1980). These workers suggested that phytosterols produce their effect through enhanced rate of cholesterol turnover and excretion as bile.

Grains A significant reduction of 10% in the level of egg yolk cholesterol was observed when triticale was substituted for wheat, however, a significant decrease could not be demonstrated when either barley or maize was substituted for wheat (McDonald and Shafey, 1989).

Garlic and Lucerne McDonald and Shafey (1989) found that when hens were fed fish oil and garlic mixed with some wheat, up to a 7% reduction in egg cholesterol was achieved. However, others found no influence on egg cholesterol concentration on modifying the hen's feed either by dietary lipid treatment or by the addition of garlic oil (Reddy *et al.*, 1991, Watkins and Elkin, 1992).

Significant reductions in yolk cholesterol were obtained following the addition of lucerne meal to a maize-based diet (McDonald and Shafey, 1989). However, the addition of lucerne and α-ketoisocaproic acid (KIC) to laying hen diets failed to consistently reduce egg yolk cholesterol levels.

Fatty Acid Content Various attempts have been made to reduce the cholesterol level in the egg by modifying the fatty acid content in the hen's diet. In Australia fat-modified eggs called Veggs are marketed. These eggs contain less saturated fat as a result of feeding hens a specially formulated fat modified natural vegetarian diet. The replacement of oleic acid in the egg with linoleic acid is facilitated by vegetable oil feeding with the result that the lipids in the egg yolk become more unsaturated. Feeding hens high fat diets may result in inflated cholesterol by stimulating the synthesis of cholesterol in the liver and deposition in the egg (Washburn and Nix, 1974a).

The addition of unsaturated oils to the diet has consistently increased yolk cholesterol (McDonald and Shafey, 1989). Beyer and Jensen (1989b) found the addition of animal by-products (mostly meat meal) to the hen's diet, as a source of dietary cholesterol, did not increase the cholesterol content of eggs, compared to those flocks which were not fed animal by-products.

The presence of highly unsaturated fatty acids in the diet increases the absorption of dietary cholesterol and increases the yolk cholesterol while saturated fatty acids have little effect (Sim and Bragg, 1978). Part of the effect of unsaturated fatty acids may be related to fat and cholesterol transport from the liver. Phospholipid synthesis has been shown to be enhanced by unsaturated and unaffected by saturated fatty acids (Hargis, 1988).

No change in egg cholesterol content was observed by Edwards *et al.* (1962) upon feeding diets containing either corn oil, beef tallow, or lard at a level of 10%. Combs and Helbacka however, observed an increase in the cholesterol content of egg yolk upon feeding a diet containing corn oil at a level of 10%, but not when they fed a diet containing animal tallow at the same level (Weiss *et al.*, 1964).

Chicks exhibit an increased turnover rate of plasma cholesterol when fed a high protein diet. This is associated with an increased faecal excretion of neutral steroids and bile acids (Yeh and Leveille, 1972). When fed a low-fat laying diet, total faecal steroids is much lower than that contained in the egg, however, when diets are supplemented with unsaturated fats, faecal steroid excretion can increase to levels close to those found in the egg (Sim *et al.*, 1980).

Dietary Fibre Dietary fibre influences cholesterol metabolism by (1) decreasing absorption of dietary cholesterol, (2) binding with bile salts in the intestinal tract, (3) shortening intestinal transit time and (4) increasing faecal excretion (McDonald and Shafey, 1989).

Yolk cholesterol concentration may be reduced by as much as 15% by choosing a suitable source of fibre. Inclusion of fibre in the diet also results in significant changes in the levels of some nutrients which may affect cholesterol metabolism, eg., changes in the level and type of fat, metabolisable energy or phytosterols (McDonald and Shafey, 1989).

Additives such as cellulose, alfalfa meal, oat hulls, cellulose and pectin or DEAE Sephadex to diets, based on either wheat and soybean meal or corn and soybean meal, were investigated (Turk and Barnett, 1972). Alfalfa added to corn-soy rations was the most effective of the fibre sources tested by Turk and Barnett (1972) with least loss of egg size, feed efficiency and egg production. The DEAE sephadex additive resulted in slightly lower egg cholesterol content than found when a conventional corn-soy layer ration was fed.

Feeding alfalfa meal, oats, sunflower meal, rice mill feed, or wood shavings to laying hens significantly decreased egg cholesterol levels when compared to those of hens fed a basal diet of corn-soybean meal. The greatest reduction was reported on feeding either oats or wood shavings up to 2% added fibre (McNaughton, 1978). This was achieved without adversely affecting either egg production or egg weight.

Oat hulls and pectin most effectively reduced cholesterol in wheat diets, but also decreased feed efficiency and egg size (Turk and Barnett, 1972). When corn diets that included DEAE sephadex were fed, egg production, feed efficiency, and egg size tended to be improved, and cholesterol concentration and cholesterol per egg were lower (Turk and Barnett, 1972). When sunflower meal was used as the fibre source a linear decrease in egg cholesterol was correlated with increasing levels of the dietary fibre (McNaughton, 1978). Leghorn pullets fed on basal layer ration containing 30% oat bran and 3% cotton seed hulls lowered ($p<0.05$) egg yolk cholesterol concentration 6 to 7% (Lirette *et al.*, 1993).

Nutrient Density Reducing the nutrient density of the diet was found to reduce yolk cholesterol. This effect could also be due to the increased fibre content of the low nutrient density diets, or to a reduction in weight gain by the hen, hence a reduction in lipogenesis. Varying the dietary levels of choline and/or methionine, which influence the lipid transport systems within the hen, did not produce a significant change in the egg cholesterol level (McDonald and Shafey, 1989).

Vitamins It has been suggested that several vitamins, such as vitamin A, niacin and ascorbic acid, reduce plasma cholesterol in the hen when added above normal levels (Hargis, 1988), however corresponding reductions in yolk cholesterol were not demonstrated. In fact, ascorbic acid reduced plasma cholesterol and raised the yolk level (McDonald and Shafey, 1989).

The zinc to copper ratio in the diet is thought to be a possible factor in determining the plasma level of cholesterol (Klevay, 1973), however, hens fed normal and low energy practical diets containing varied ratios of zinc and copper showed no

significant differences in levels of either plasma or egg cholesterol (Helwig *et al.*, 1978).

Supplementing the diets of white leghorn layers with 100mg and 150mg probiotic per kg of food reduced egg yolk cholesterol concentrations from 14.69mg to 11.28 and 11.37mg/g, respectively. Serum cholesterol values were similar between controls and treated birds when diets were supplemented with 100mg/g probiotic but decreased from 176.5mg/dL to 114.3mg/dL when 150mg/g probiotic was added to the diet (Mohan *et al.*, 1995).

Bacteria A selected strain of Lactobacillus acidophilus when fed to laying hens at 4 million viable cells/g of feed reduced the cholesterol values in yolk by as much as 18.8% compared to control flocks. This was thought to be a possible result of a dramatic drop in serum cholesterol of 55% after the bacillus was added to the feed (Haddadin *et al.*, 1996).

In summary, attempts to consistently reduce egg cholesterol levels by either altering the laying hen's diet, or through selection programs have only been marginally successful. The relative resistance of egg composition to alterations in diet apparently reflects the nutritional and structural requirements for avian embryonic development (Kuksis, 1992). Although dietary intake has been shown to influence egg cholesterol levels (Sutton, 1984, Sim *et al.*, 1980), Beyer and Jensen (1989a) found that the cholesterol level of market eggs remained the same irrespective of the feed source

There have been many attempts to reduce the cholesterol content of eggs but most have demonstrated that the cholesterol level is difficult to alter in the domestic fowl. Another approach to lowering egg cholesterol is the administration of pharmacological agents to laying hens.

1.7.3 Pharmaceuticals

During the past 25 years, many drugs that have been employed as hypercholesterolaemic agents in humans have been orally administered to chickens (Naber, 1983; Hargis, 1988). These drugs alter cholesterol metabolism, by interfering with either cholesterol synthesis or sterol excretion (Naber, 1983).

Examples of hypercholesterolaemic agents used are ethyl p-chlorophenoxyisobutyrate (CPIB) and D-thyroxine (Weiss *et al.*, 1967b). Both CPIB and D-thyroxine injected subcutaneously reduced blood cholesterol but increased egg cholesterol concentration, with higher dosages having an adverse affect on egg production (Weiss *et al.*, 1967a,b). Oral administration of D-thyroxine also increased the yolk cholesterol content, but did not produce the same decrease in plasma cholesterol. Secondary effects of D-thyroxine also included decreased yolk size and moulting (Weiss *et al.*, 1967a,b).

Others noted that 80% of the cholesterol in the yolk was replaced with desmosterol after feeding Triparanol, (Naber *et al.*, 1982; Burgess *et al.*, 1962), and that Probucol reduced yolk cholesterol by 4 to 7% when fed at a level of 0.1% of the diet (Naber *et al.*, 1982).

Recent work by Elkin and Roger (1990), with the human hypercholesterolaemic drug lovastatin, suggested that HMG-CoA reductase inhibitors hold promise as effective egg cholesterol lowering agents. When PD 123244-15 [(±)-(R*,R*)-3,4-dibromo-β,δ-dihydroxy-2-(4-fluorophenyl)-5-(1-methylethyl)-1H-pyrrol-1-heptanoic acid, sodium salt], a totally synthetic HMG-CoA reductase inhibitor, was fed to laying hens the egg cholesterol content varied inversely with the amount of dietary PD 123244-15, with a maximum reduction of approximately 30% obtained at the highest drug level (Elkin *et al.*, 1993). No drug residues were detected in either egg yolk or albumin extracts. Nevertheless, even if the Food and Drug Administration were to approve the procedure, Lovastatin is too expensive to use in commercial egg production (Crawford, 1990).

Little effect was found on yolk cholesterol levels even after inhibiting 43% of the activity of HMG-CoA reductase using 7-ketocholesterol (Vargus *et al*, 1986). The biological half life of plasma cholesterol in the hen is 36h, compared to 60h in rats (Andrews *et al.*, 1968). This indicates a far more rapid metabolism in the former species. Thus, the hen has a capacity to synthesise considerably more cholesterol than it normally produces.

Research into the effects of cholesterol-lowering using drugs fed orally or injected have often resulted in cessation of egg production or an accumulation of undesirable metabolites in the egg (Elkin *et al.*, 1993). With current technology it appears unlikely that dietary changes, drugs or selective breeding will produce changes to egg cholesterol levels of significance to the human diet (Naber, 1983).

1.7.4 Removal of Egg Yolk

Egg marketing authorities of Victoria and Queensland have developed and marketed "Scramblers" and "Yolk Free", respectively, low cholesterol foods containing largely egg albumin and vegetable oil. Egg yolk is partially replaced with vegetable oil or water then combined with egg white (Anon., 1991; Cully and Vollbrecht, 1992).

Trained flavour profile scores for scrambled eggs were shown to decrease as the level of egg white was increased. Although egg white contributes greatly to scrambled egg flavour by producing the very low threshold sulphur compounds, the yolk influences the scrambled egg flavour through the sweet note, and may be involved in retention of other flavour components (Warren and Ball, 1990).
Thus, many recombined egg products may fail to retain the desirable egg flavour demanded by consumers.

1.7.5 Removal of Cholesterol- Containing Fat Components

Other methods used to reduce cholesterol involve removing the cholesterol-containing fat components. These methods have been effective in skim or low-fat milk. Unfortunately, the low-fat product may be organoleptically different from the full-fat product. Some food products produced may also depend upon the fat to provide function and bulk (Hegenbart, 1989). Manufacturers are then faced with the problem of having to replace not only the bulk, but also the flavour and functional qualities of the missing fat.

A need has now been created to develop methods for reducing the cholesterol content in egg products without lowering organoleptic and functional properties.

Novel Food Ingredients in the Reformulation of Food Products

Intensive research and development efforts have been directed towards the development of novel food ingredients to be employed in the reformulation of food products (Gross, 1991).

Fat in cholesterol-reduced butter has been replaced with water and dairy proteins to literally dilute the cholesterol content. One disadvantage of this product is that it cannot be used for pan frying because it is likely to splatter. Some cheese manufacturers are also using milk proteins to replace cholesterol-containing butterfat in cheese products.

Emulsifiers have been used to replace the functional properties of fat, but not the bulk. These help to bind a product together and maintain the lubricity of mouthfeel that is normally lost when fat levels are reduced. In the baking industry cholesterol -containing fat is replaced on a 1:1 basis with a no-cholesterol fat. Manufacturers of frozen novelties, are replacing tropical oils in ice-cream products with soybean oil, for example, which does not increase cholesterol levels. However, these fat-replacers are awaiting Food and Drug Administration (FDA) approval, which may take several years (Hegenbart, 1989).

For traditional egg-based products, the lack of normal egg flavour and the poor flavour performance of egg substitutes, may be cause for complaints by consumers

(Warren and Ball, 1990). Many research teams are seeking to find a way to remove cholesterol directly from the fats that contain it, thus eliminating many of the concerns involved with developing organoleptically acceptable products (Hegenbart, 1989).

1.7.6 Extraction Processes

Steam Distillation

Steam stripping used to deodorise fish oils also removes cholesterol from fish oil and other fats. Up to 93% of the cholesterol is removed while yielding up to 95% recovery of fat. In the process of steam stripping oil, temperatures as high as 220-290°C are used to vaporise the oil into a chamber (Hegenbart, 1989). It is most unlikely that whole egg pulp or egg yolk could withstand such a treatment without suffering organoleptic deterioration. Both steam-stripping of cholesterol and fat-replacing techniques would also add cost to the final product.

Organic Solvents

Extraction of egg cholesterol using organic solvents has met with limited success. A large amount of cholesterol remains in the extracted egg, and the functional properties of the egg yolk are affected (Lebovics *et al.*, 1992). Organic solvents remove phospholipids and denature proteins, both of which are responsible for functional properties (Froning *et al.*, 1990).

Using hexane and isopropanol as extraction solvents, Larsen and Froning (1981) fractionated egg yolk into an egg oil, a protein isolate and an aqueous component. It was suggested that the egg oil fraction could be used as a cooking oil and in pharmaceutical products and cosmetics, and the protein isolate as a food binder and emulsifier.

The egg oil fraction contained the highest concentration of cholesterol, which was removed (up to 40%) by degumming, refining and bleaching. Analysis of the crude refined egg oil also showed a decrease in the ratio of unsaturated to saturated fatty acids as compared to intact egg yolk (Larsen and Froning, 1981).

Emulsion stability tests of the fractions revealed that neither was stable. Hexane greatly affected the emulsion stability by altering the protein integrity. When used to make mayonnaise, the viscosity associated with these emulsions were not as high as those found with mayonnaise using native egg yolk (Larsen and Froning, 1981). Also, in a sensory evaluation, panelists detected significantly more solvent odour in egg oil samples compared to corn and soybean oil. Egg oil also had high levels of rancidity (Larsen and Froning, 1981).

The development of egg oils has been described previously in literature (Romanoff and Romanoff, 1949; Anon., 1973). However, the production of these oils requires diethyl ether or chloroform extraction, which is unlikely to be acceptable for food products (Larsen and Froning, 1981).

Supercritical Fluid Extraction

Capitalising on the unique properties of solvents above their critical conditions to extract target compounds from mixtures is the basic feature of a relatively new extraction and purification process known as supercritical fluid extraction (SFE).

An SFE system is made up of four basic components: a compressor, an extraction vessel, a temperature and pressure control unit and a separation vessel. An extraction solvent can be any liquid in a supercritical state. In such a system, a mixture is placed in contact with a supercritical extraction fluid in the extraction vessel. After the extraction, the supercritical fluid containing the extractives is introduced into the separation vessel and separated into product and solvent by reducing pressure. The solvent can be compressed and re-used. The system can be operated under different conditions to extract the target products.

Although there is some concern over the economics of SFE (Hegenbart, 1989), in spite of the high capital investment required, the process already has several commercial applications in the food industry.

Supercritical carbon dioxide is currently being used to extract flavours and decaffeinated coffee (Hegenbart, 1989). Food scientists are also investigating such applications as the extraction of spices, removal of oil from snack foods, extraction of oil from cotton seed, corn and soybeans, and extraction of oil from full-fat soy (Froning *et al.*, 1990). Smaller scale successes have been achieved with fluid milk and butter (Hegenbart, 1989). Supercritical carbon dioxide has also been used to extract carotene and lutein from leaf protein concentrates (Favati

et al., 1988). In other products, cheese for example, SFE may not be feasible as it could alter the flavour, texture and colour of the product (Best, 1989).

In eggs, up to 80% of egg cholesterol can be removed using carbon-dioxide as the extraction fluid. However, an economical technique to recover the cholesterol from the significant amount of co-extracted egg lipid is required as a prerequisite for commercial feasibility (Froning *et al.*, 1990; Hung and Unger, 1993).

Froning *et al.* (1990) managed to extract two-thirds of the cholesterol from dried egg yolk at 306atm/45°C or 374atm/55°C compared to 163atm/40°C or 238atm/45°C. The authors explained that this was due to the increased fluid density and greater volatility of cholesterol at the higher temperature/pressure conditions.

Phospholipids and protein were concentrated at the highest supercritical extraction conditions (374atm/55°C) used, due to the significant reduction of total lipids (36%) under these conditions. This was a desirable effect as the phospholipids are necessary for maintenance of good functional properties.

When used in sponge cake batter, SFE extracted egg yolk powder significantly improved sponge cake volume. This was observed at all extraction pressures and temperatures studied by Froning *et al.* (1990) except at 374atm/55°C, ie. 163atm/40°C; 238atm/45°C and 306atm/45°C. The emulsion stability of mayonnaise, prepared with SFE extracted egg yolk powder was adversely affected by the 374atm/55°C extract, however the performance of yolk extracted at the milder treatment of 306atm/5°C was not significantly different from the control,

generally indicating that supercritical extraction of cholesterol can be utilised without substantially impairing the stability of mayonnaise (Froning *et al.*, 1990).

Extraction at increased temperatures and densities produced a dried egg yolk of lighter colour with less redness and yellowness indicating removal of some of the xanthophyll pigments (Froning *et al.*, 1990).

Another undesirable effect of SFE is the increasing saturated to unsaturated fat ratio (S/U), as the temperature and/or pressure is increased (Froning *et al.*, 1990). This was considered by Froning *et al.* (1990) to be a possible reflection of the concentration of the phospholipids in the extracted egg yolk powder rather than any specific fatty acid selectivity, since the extraction conditions used removed the majority of triglycerides in the system.

This increase in S/U fat may therefore, be of concern to health-conscious consumers who may be increasing their background diet of saturated fats even when consuming the low-cholesterol egg product (Froning *et al.*, 1990).

Nonetheless, SFE extraction technology has excellent potential for production of a lower cholesterol, lower fat egg product.

Cyclodextrin

A natural, non-toxic compound derived from starch, β-cyclodextrin is composed of a ring of seven glucose units linked together by α-1,4 glycosidic bonds (Bender, 1986). Cyclodextrin molecules can bind other compounds in their

cavity, thereby stabilising, solubilising, or precipitating the compounds (Pszczola, 1988). When added to egg pulp, β-cyclodextrin forms an insoluble complex with cholesterol and cholesteryl ester.

The potential of cyclodextrin as an effective cholesterol removal reagent, was discovered by chance by Sidhu and Oakenfall, when investigating the absorption inhibiting effect of saponins on cholesterol in the gut for lowering plasma cholesterol (Davidson, 1990).

Cyclodextrin, unlike other proposed cholesterol-reduction technologies, is a cheap alternative, not requiring expensive and specialised equipment, and because it can be used at low temperatures, minimises the risk of microbiological spoilage or loss of flavour volatiles. Removal of up to 90% egg cholesterol has been described using this process (Cully and Vollbrecht, 1991).

β-cyclodextrin treated egg yolk was found to produce a low cholesterol egg product with similar compositional and functional properties similar to untreated egg yolk. The cholesterol reduced egg yolk contained less lipid and protein and more carbohydrate and ash than control egg yolk. Free and esterified cholesterol was reduced by 91.6% and 94.4%, respectively (Awad *et al.* 1997).

Unfortunately, being a somewhat cumbersome batch process it inevitably leaves residues of the complexing agent in the product (Davidson, 1990). Even with an additional enzymatic treatment it is still difficult to completely remove all residues of β-cyclodextrin from the yolk (Cully and Vollbrecht, 1991).

Though established as an acceptable food additive in a number of European countries and in Japan, β-cyclodextrin has yet to gain approval as a food additive by authorities in some countries, including the United States and Australia (Cully and Vollbrecht, 1991). This may also prove to be a major obstacle to the widespread use of the agent.

Various attempts have been made to solve the problem of β-cyclodextrin residue. For example, it has been suggested by Sidhu and Oakenfall that the molecule be chemically bonded to a support, such as a mass of silica beads; the adsorbent then used to extract cholesterol from milk or egg, and the beads washed for re-use (Davidson, 1990). However, more research is needed to optimise the process and to develop an economically viable and practical route for the production of low-cholesterol products (Davidson, 1990).

Consequently, cholesterol reduced egg products continue to be prepared by rather complex processes. There is a need for a simple technique to produce cholesterol free or cholesterol reduced egg yolk, without the loss of egg flavour, as a food ingredient for a variety of egg-based products.

1.7.7 Enzymatic Degradation

Industrial Significance of Enzymes

The food industry is the largest user of enzymes. Kindel (1981) has reported more than 2,500 enzymes being officially recognised by the International Union of Biochemistry, speculating that 25,000 natural enzymes exist (Neidleman, 1991).

The world-wide market for industrial enzymes had an estimated value of $625 million in 1990, with the U.S. as the largest sector; approximately 62% of the enzymes produced were destined for food applications (Leblond, 1990).

Enzymes can be used in the production of value-added ingredients and speciality chemicals using a variety of biocatalytic reactions, however the commercial development of such processes is often limited by the difficulties associated with the development of multi-enzyme reactions needed to achieve the desired final product (Penet, 1991).

In the food industry the largest use of enzymes is in starch processing followed by cheese production, fruit and vegetable juice, and barley and brewing (Leblond, 1990). Enzymes have also been used to create functional properties from deamidated soy protein (Hamada and Marshall, 1989), in the transesterification of unsaturated lipids by lipases (Osterberg *et al.*, 1989), and for enhancing the functional properties of casein with trypsin (Chobert *et al.*, 1988).

Today plants, animals and microbes are all commercial sources of useful industrial enzymes. Enzymes such as α- and β-amylases, proteases, amyloglucosidase, pentosanase, glucanase, and phytase used in bakery processing come from fungi and bacteria; the fungal genera *Aspergillus, Rhizopus* and *Trichoderma* are the chief sources of enzymes used in fruit and juice processing; chymosin, used by the dairy industry to coagulate milk, is obtained by saline extraction of the abomasum, or fourth stomach, from unweaned calves; the fungi, *Mucor miehei, Mucor pusillus* and *Endothia parasitica* are sources of chymosin-like proteases used as commercial substitutes for calf rennet in the manufacture of cheese; and

commercially available lactase preparations are commonly purified from the yeast *Kluyveromyces lactis* (formerly *Saccharomyces lactis*) and the fungi *Aspergillus oryzae* (Dziezak, 1991).

Other examples which illustrate the potential of enzymes as catalysts in the production of food ingredients are the use of a lipase in the regio-specific transformation of fats and oils to produce β-monoglycerides and the use of cholesterol reductase to convert cholesterol into a non-toxic compound (Gross, 1991).

Immobilisation of Enzymes

The stability of enzymes is important for the economy of their industrial applications since highly purified enzyme preparations are expensive.

Storage stability depends upon the physical form of enzyme preparations and solid forms generally have higher stability than liquid forms (Randolph *et al.*, 1988). Immobilisation is a means of creating a solid enzyme form and essentially eliminates the danger of proteolysis, however the risk of microbial degradation is higher. Immobilisation of an enzyme onto a solid support allows processors to take advantage of the enhanced reusability of this enzyme form (Dziezak, 1991). The continuous process of lactose hydrolysis relies on lactase that has been immobilised on a solid support such as a column-type reactor (Dziezak, 1991).

1.7.8 Microbiological Degradation

Work done by Watanabe *et al.* (1986) suggested that cholesterol decomposing microorganisms in nature existed not only among *Mycobacterium* and *Nocardia* species as had been previously recognised but were also widely distributed among actinomycetes and bacteria.

Cholesterol-degrading bacteria (16 strains) have been isolated from foods of animal origin such as butter, bacon, pork fat and chicken fat. Most have been identified as *Rhodococcus equi* or *Rhodococcus erythropolis* (Ferriera and Tracey, 1984; Watanabe *et al.*, 1986). These cholesterol-degrading bacteria are known to use cholesterol as the sole source of carbon for growth and have been found to have similar cholesterol-degrading ability but with some differences in the production of cholesterol oxidase (COD) (Aihara *et al.*, 1986; Watanabe *et al.*, 1986).

Microbiological degradation of cholesterol by some strains of *Nocardia*, *Brevibacterium*, *Corynebacterium* or *Streptomyces* have also been reported (Aihara *et al.*, 1986, 1988a,b). Some strains of these organisms accumulate steroid intermediates, with consequent adverse health effects, however other strains which can degrade cholesterol without accumulating steroid intermediates have also been isolated (Aihara *et al.*, 1988b).

Isolated intracellular and extracellular COD of various strains of cholesterol-degrading bacteria degraded over 70% of the cholesterol in a pure cholesterol preparation within 3 days (Watanabe *et al.*, 1986). Johnson and Somkuti (1990)

also found *Rhodococcus* strains could reduce up to 80% of cholesterol in a pure cholesterol preparation after 7 days growth. Although these findings indicate that enzymatic treatment could be used for egg cholesterol degradation, for any commercial application it would be necessary to shorten the degradation time of 3 days reported in these studies.

1.8 Project Aims and Objectives

The overall objective of this project is to define the parameters affecting the production of low cholesterol egg yolk products by enzymatic means.

This will include the following specific aims:

- the development of a rapid and simple method for the assay of cholesterol in egg yolk and other egg-based food products,
- the investigation of the cholesterol-degrading ability of cholesterol reductase and COD in egg yolk and
- the cholesterol-degrading activities of *R. equi*, *R. erythropolis* and two other unspeciated isolates of *Rhodococcus* .

CHAPTER 2

Materials and Methods

2.1 Cholesterol Assay Conditions

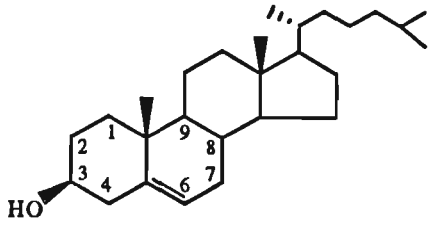
Solvents

- Absolute ethanol, analytical grade, Sigma Chemical Co.
- Chloroform, HPLC grade, Ajax Chemicals.
- Diethyl ether, laboratory reagent, Ajax Chemicals.
- Hexane, laboratory reagent, Ajax Chemicals.
- Methanol, Absolute, Sigma Chemical Co.
- Petroleum ether, AnalaR, BDH Chemicals, Australia Pty Ltd.

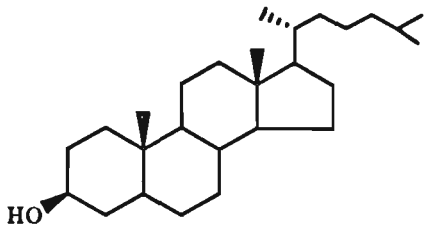
Chemicals

- di-potassium hydrogen orthophosphate, analytical reagent, BDH Chemicals.
- di-Sodium hydrogen orthophosphate, analytical reagent, BDH Chemicals.
- N-methyl-N-(trimethylsilyl)-trifluoroacetamide $C_6H_{12}F_3NOSi$ (MSTFA), Sigma Chemical Co.
- N-O-bis-(trimethylsilyl)-trifluoroacetamide $C_8H_{18}F_3NOSi_2$ (BSTFA), Alltech (Aust) Pty. Ltd.
- Potassium dihydrogen orthophosphate, analytical reagent, BDH Chemicals.
- Potassium hydroxide, analytical reagent, BDH Chemicals.
- Sodium dihydrogen orthophosphate, analytical reagent, Ajax Chemicals.

Chromatography Standards



- Cholesterol, (Cholest-5-en-3β-ol) $C_{27}H_{46}O$, mol wt 386.64g/mol, 99 %, Sigma Chemical Co.



- Dihydrocholesterol, (3β-hydroxy-5α-cholestane), $C_{27}H_{48}O$, mol wt 388.65g/mol, approx. 95%, Sigma Chemical Co.
- Octacosan-1-ol ($C_{28}H_{58}O$), mol wt 410.74g/mol, approx. 99% by capillary GC, Sigma Chemical Co.

Instruments

Hewlett Packard 5890 Gas Chromatograph

Column :	BP-10, 25m x 0.25mm i.d.
Phase thickness:	0.25μm
Injector mode:	split ratio 30:1
Injection volume:	1μL in chloroform
Detector:	FID or MSD Hewlett Packard 5971A Mass Selective Detector

Temperatures:	Injector	250°C
	Detector	320°C
	Oven	285°C isothermal
Carrier gas:	0.8mL/min He	
Acquisition mode:	scan	
Scan rate:	1.1/s	
Scan range:	40-550amu	
Run time:	12min	
Work station:	Hewlett Packard Vectra 386/25	
Software:	HP ChemStation	

Varian 3700 Gas Chromatograph

Column :	BP-10, 25m x 0.25mm i.d.	
Phase thickness:	0.25µm	
Injector mode:	split ratio 50:1	
Injection volume:	0.5µL in chloroform	
Detector:	FID	
Temperatures:	Injector	300°C
	Detector	350°C
	Oven	285°C isothermal
Carrier gas:	0.8mL/min He	
Run time:	10min	
Range:	10^{-11} A/mV	
Integrator:	Shimadzu CR-1B Chromatopac	
Chart speed:	0.5cm/min	

2.1.1 Cholesterol Determination

Cholesterol was determined using a modification of the method described by Kovacs (1990).

A 0.1g sample of egg yolk, either weighed directly or dispensed as a 0.5mL aliquot of a 0.2g/mL dispersion in 0.1M phosphate buffer (pH7), was placed into a 15mM x 100mM test-tube fitted with a teflon-lined screw cap. 1M KOH in absolute ethanol (2mL), containing 0.5mg/mL of internal standard, dihydrocholesterol, was added and the sealed tube heated at 100°C for 1h. After the digestion, diethyl ether (3mL) and water (2mL) were added, the mixture was vortexed and the ether phase taken into a second test-tube fitted with a teflon-lined screw cap. The aqueous phase in the first test-tube was extracted with a further 2mL diethyl ether. A few drops of saturated NaCl solution was required to avoid emulsion formation. The ether phase was combined, washed three times with water (1mL) and then passed through a 4mmx25mm column of anhydrous Na_2SO_4, to remove moisture, into a 1.5mL glass reaction vial. The ether was evaporated in a stream of N_2 at 40°C, 100µL MSTFA added and the sealed vial heated at 100°C for 15min. The reaction product was diluted with 500µL $CHCl_3$ and 1µL taken for gas chromatography using the Hewlett Packard gas chromatograph and the conditions outlined in Chapter 2.1. Under these chromatographic conditions, retention times of the TMS derivatives of cholesterol and dihydrocholesterol were typically 10 and 10.3min respectively.

The identification of cholesterol and dihydrocholesterol in gas chromatography traces was facilitated by comparison of retention times with those of known standards chromatographed under identical conditions. Quantification of

cholesterol was achieved by comparison of the areas of the cholesterol and dihydrocholesterol peaks and the application of a small factor to recognise a slight difference in the response of each of these compounds in the flame ionisation detector.

2.1.2 Cholesterol Content in Multi-component Foods

A survey of cholesterol content of several typical western foods and their oriental analogues, available in Melbourne markets, was conducted using the method described in section 2.1.1. with the FID fitted to the gas chromatograph. Fresh noodles were milled in a Bamix® miller, dried overnight at 100°C, and milled again. Other foodstuffs were also milled in a Bamix® miller, except pork cake which was mixed with a glass stirring rod.

A total of 15 Western foods and 10 Asian foods were assayed for cholesterol, and data was expressed as average mg cholesterol/g sample±s.e (n=3).

2.2 Optimisation of Cholesterol Assay Conditions

2.2.1 Internal Standards

In order to compare the effectiveness of the two internal standards used in this work, a 0.1g sample of fresh egg yolk was assayed for cholesterol using either dihydrocholesterol or octacosan-1-ol.

2.2.2 *Substrate*

A 0.2g/mL suspension of egg yolk was prepared in 0.1M pH7 phosphate buffer, and 0.5mL aliquots assayed for cholesterol (n=4). The same egg yolk was assayed fresh (0.1g) (n=4).

2.2.3 *Silylation*

A number of silylating agents are available for the derivatisation of the hydroxyl groups of steroids. The two reagents studied for the formation of trimethylsilyl (TMS) ether derivatives of cholesterol and the internal standards were N-O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) (Figures 2.1 and 2.2). The silylated structures for cholesterol and internal standard dihydrocholesterol are shown in Figures 2.3 and 2.4, respectively. The formula for silylated octacosan-1-ol is $C_{28}H_{57}OSi(CH_3)_3$.

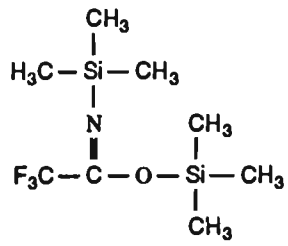


Figure 2.1 The structure of BSTFA (N-O-bis-(trimethylsilyl)-trifluoroacetamide), $C_8H_{18}F_3NOSi_2$, Alltech (Aust) Pty. Ltd.

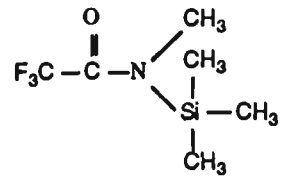


Figure 2.2 The structure of MSTFA (N-methyl-N-(trimethylsilyl)-trifluoroacetamide), $C_6H_{12}F_3NOSi$, Sigma Chemical Co. and Alltech (Aust) Pty. Ltd.

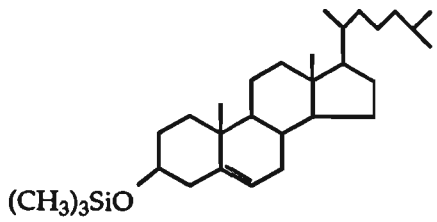


Figure 2.3 The structure of MSTFA silylated cholesterol, $C_{27}H_{45}OSi(CH_3)_3$.

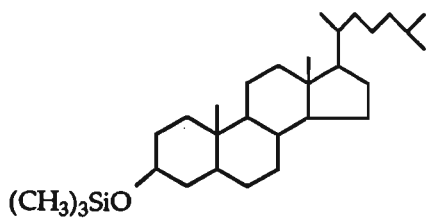


Figure 2.4 The structure of MSTFA silylated dihydrocholesterol, $C_{27}H_{47}OSi(CH_3)_3$.

Silylation Reaction Time Studies

A study of the silylation reaction was conducted in order to establish conditions necessary for complete derivatisation. A mixture containing equal amounts of cholesterol and octacosan-1-ol (1mg/mL) in chloroform was prepared. The mixture (1mL) was dispensed into septum-stoppered glass vials, evaporated to dryness under a stream of nitrogen, and derivatised with silylating agent (either BSTFA or MSTFA) for varying time periods (0, 1, 2, 3, 5, 10, 20, 30, 40, 50 and 60min) with intermittent shaking at room temperature. A four fold excess of silylating reagent (100µL) was routinely used for these reactions. Each sample was diluted with 100µL chloroform, and 0.5µL taken for gas chromatography using the equipment and conditions outlined in section 2.1.

2.2.4 Gas Chromatography

Position of Capillary Column in Injector of Varian Gas Chromatograph

1mL aliquots of a mixture containing equal amounts of cholesterol and octacosan-1-ol (1mg/mL) was prepared, and were evaporated to dryness in septum-stoppered glass vials and derivatised with excess MSTFA (100μL) in preparation for gas chromatography.

The capillary column was inserted 11cm into the injector port liner of the Varian gas chromatograph and progressively lowered until 4cm remained inserted. At each centimetre interval, a 0.5μL injection of the standard mixture of derivatised cholesterol and octacosan-1-ol in chloroform (1mg/mL) was made. The peak area ratios and peak heights of the standards were recorded.

Relative Response Factors

One millilitre aliquots of a mixture containing equal amounts of cholesterol and each internal standard were evaporated to dryness at 40°C and then derivatised with excess MSTFA in preparation for gas chromatography.

The response factor for each of the internal standards was determined in relation to cholesterol by measuring the peak area of the internal standard relative to cholesterol.

2.2.5 Reproducibility and Recovery

Reproducibility and recovery data were collected for both egg yolk and egg-based food products. The yolks of three eggs were combined and mixed until homogeneous and 0.1g samples of the homogenate were taken for testing. Uncooked egg noodles, purchased locally, were ground to uniformity using a Bamix® mill and 0.1g samples taken for testing.

For the comparison of cholesterol values obtained from the original and modified extraction procedure of Kovacs, replicate samples (eight and seven, respectively) were taken and carried through each procedure. The reproducibility was expressed as a percentage coefficient of variation and the mean cholesterol was expressed as mg cholesterol/g sample±s.e.

The efficiency of recovering cholesterol by each extraction procedure was tested using replicate samples (eight and seven) to which a known amount of pure cholesterol had been added; cholesterol dissolved in ethanolic potassium hydroxide solution (1M) was added to each sample prior to saponification. The % recovery was determined by subtracting the average peak area of cholesterol present in unspiked samples from that of the spiked samples.

2.3 Enzymatic Treatment of Egg Yolk

Organic Material

- Cucumber leaves (3 week old)

Enzymes

- Cholesterol oxidase derived from *Brevibacterium species* (Bs), lyophilised powder, Sigma Chemical Co.
- Cholesterol oxidase derived from *Nocardia erythropolis* (Ne), lyophilised powder, Sigma Chemical Co.
- Cholesterol oxidase derived from *Pseudomonas fluorescens* (Pf), lyophilised powder, Sigma Chemical Co.
- Cholesterol oxidase derived from *Streptomyces species* (Ss), lyophilised powder, Sigma Chemical Co.

Solvents

- Absolute ethanol, analytical grade, Sigma Chemical Co.
- Acetonitrile, ACS reagent, Sigma Chemical Co.
- Chloroform, HPLC grade, Ajax Chemicals.
- Diethyl ether, laboratory reagent, Ajax Chemicals.
- Isopropanol anhydrous, Sigma Chemical Co.
- Methanol, Absolute, Sigma Chemical Co.

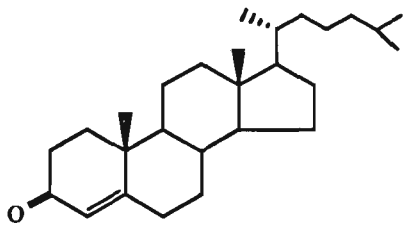
Chemicals

- Amberlite resin, XAD-4, Sigma Chemical Co.
- Ammonium nitrate, analytical reagent, Mallinckrodt Australia.
- Ascorbic acid, analytical reagent, BDH Chemicals.

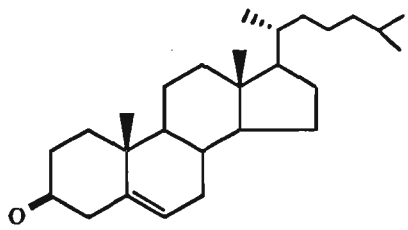
- 2', 7'-dichlorofluorescein, spray reagent, 0.2% in ethanol, Sigma Chemical Co.
- Dithiothreitol, 99+%, Sigma Chemical Co.
- Ethylenediaminetetraacetic acid, analytical reagent, Ajax Chemicals.
- Glycerol, 99+%, Sigma Chemical Co.
- di-potassium hydrogen orthophosphate, analytical reagent, BDH Chemicals.
- di-sodium hydrogen orthophosphate, analytical reagent, BDH Chemicals.
- Ferrous sulphate heptahydrate, 'Baker analysed' reagent, J. T. Baker Chemical Co.
- Liquid nitrogen, Liquid Nitrogen Services Pty. Ltd.
- Magnesium sulphate, analytical reagent, BDH Chemicals.
- MSTFA (N-methyl-N-(trimethylsilyl)-trifluoroacetamide), Sigma Chemical Co.
- 2-(N-morpholino)ethanesulphonic acid, crystalline, 99+%, Sigma Chemical Co.
- Nicotinamide adenine dinucleotide phosphate (NADPH)
- Polyvinylpolypyrrolidone (PVPP), Polyclar AT, Sigma Chemical Co.
- Potassium dihydrogen orthophosphate, analytical reagent, BDH Chemicals.
- Potassium hydroxide, analytical reagent, BDH Chemicals.
- Sodium dihydrogen orthophosphate, analytical reagent, Ajax Chemicals.
- Sodium metabisulphite, ACS reagent, Sigma Chemical Co.
- Sodium sulphate anhydrous, analytical reagent, Ajax Chemicals.

- Sucrose, analytical reagent, Ajax Chemicals.
- Thimerosal, approx. 98% (HPLC), Sigma Chemical Co.

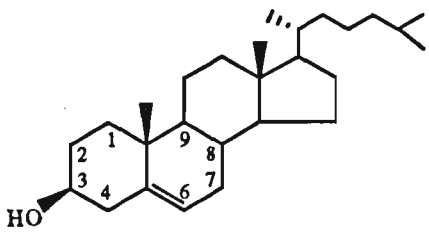
Chromatography Standards



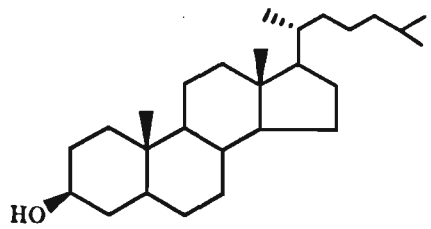
- Cholest-4-en-3-one (3-keto-4-cholestene), $C_{27}H_{44}O$, mol wt 384.6g/mol, approx. 97%, Sigma Chemical Co.



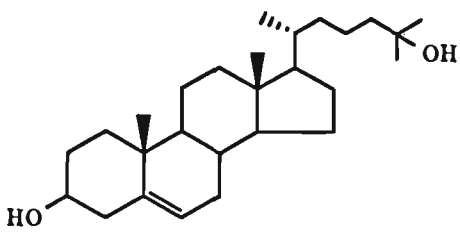
- Cholest-5-en-3-one (3-keto-5-cholestene), $C_{27}H_{44}O$, mol wt 384.6g/mol, approx. 97%, Sigma Chemical Co.



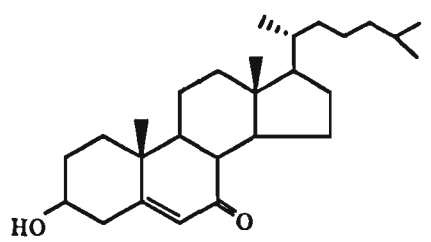
- Cholesterol (cholest-5-en-3β-ol) $C_{27}H_{46}O$, mol wt 386.64g/mol, 99%, Sigma Chemical Co.



- Dihydrocholesterol (3β-hydroxy-5α-cholestane), $C_{27}H_{48}O$, mol wt 388.65g/mol, approx. 95%, Sigma Chemical Co.



- 25-hydroxycholesterol (cholest-5-en-3β, 25-diol), $C_{27}H_{46}O_2$, mol wt 402.7g/mol, pfs, Sigma Chemical Co.



- 7-ketocholesterol (cholest-5-en-3β-ol-7-one), $C_{27}H_{44}O_2$, mol wt 400.6g/mol, minimum 90%, Sigma Chemical Co.

Egg Sample

Batches of liquid egg yolk were prepared from 61 locally purchased fresh Grade AAA average-sized eggs. Yolks were separated from albumin and blended at low speed in a domestic blender until homogeneous. A 0.2g/ml suspension of yolk in 0.1M pH7 potassium phosphate buffer containing 0.02% thimerosal as an anti-bacterial agent was prepared and 20ml portions stored at -4°C until required.

2.3.1 Cholesterol Reductase

Method of Extraction from Cucumber Leaves

A cholesterol reductase enriched solution that could be used to decrease the cholesterol concentration in egg yolk was prepared from cucumber leaves (Beitz *et al.*, 1990).

The method involved grinding 20g of young cucumber leaves (3 to 6 weeks old) in liquid nitrogen using a mortar and pestle sitting in an ice bath. Finely ground dry amberlite resin (1g dry wt/g fresh cucumber leaves) was then mixed into the frozen powder and the mixture was held in the ice bath for several minutes to allow the enzymatic release of isothiocyanates.

Homogenisation buffer (sodium/potassium phosphate buffer, 100mM, pH6.5) containing sucrose (150mM), sodium metabisulphite (10mM), ascorbic acid (10mM), dithiothreitol (5mM), and ethylenediaminetetraacetic acid (0.1mM), was slowly added to the mixture (5mL/g cucumber leaves) and proteins were extracted by stirring in insoluble PVPP (5g hydrated wt/g fresh cucumber leaves) to produce a thick slurry. Insoluble PVPP is used to remove those plant phenolic compounds which form strong hydrogen bonded complexes with proteins, however, some phenolic compounds, as well as certain other interfering secondary products, are not bound effectively by PVPP (Loomis *et al.*, 1979).

The slurry was then squeezed through four layers of gauze into a separating funnel suspended over a glass column packed with amberlite XAD-4 resin. The tap of the

funnel was opened and the filtrate allowed to pass down the column. The amberlite resin facilitated the removal, through hydrophobic interactions, of hydrophobic secondary products and surface active materials, including phenolics and contaminating monoterpenes from the plant enzyme extract (Loomis *et al.*, 1979). This procedure was repeated a second time after regeneration of the column by washing with methanol, followed with water.

The filtrate was then centrifuged at 27,000Xg (approx. 18,000rpm) for 20min at 4°C and the supernatant passed down an amberlite XAD-4 resin column to remove any persistent monoterpene contaminants.

The supernatant was centrifuged once more, this time at 140,000Xg (approx. 41,000rpm) for 1h at 4°C. The resulting supernatant (cytosol) was then dialysed against a solution of dialysis buffer: 10mM sodium/potassium phosphate buffer (pH6.5) containing 20mM 2-[N-morpholino]ethanesulphonic acid, 0.5mM dithiothreitol, and 10% (v/v) glycerol, at 4°C for 24h, with the dialysis buffer being changed at least 3 times.

The clear dialysed concentrate was employed as the cholesterol reductase-enriched preparation.

Purification and Preparation of XAD-4 Amberlite Resin

Purification of the amberlite XAD-4 resin is important before use. A purification procedure followed by Loomis *et al.* (1979) was adopted whereby approximately

30g of resin was sequentially extracted with methanol, acetonitrile and petroleum ether (100mL) in a Soxhlet extractor for 8h each.

The beads of amberlite resin were then combined and stored under methanol in a glass column. Prior to use the column was washed with water.

Purification and Preparation of PVPP

PVPP was purified by boiling for 10min in 10% hydrochloric acid and washing with glass distilled water until free of chloride. The absence of chloride was measured by adding silver nitrate ($AgNO_3$) dropwise into the boiled mixture until no colour change was observed. The washing process was hastened by neutralising with potassium hydroxide after several changes of wash water. The purified PVPP was then dried for storage and when required hydrated in homogenisation buffer for several hours before use

Cholesterol Degrading Capacity of Cholesterol Reductase Enriched Extract

The cholesterol degrading capacity of the cholesterol reductase extract was investigated with both pure cholesterol and egg yolk. Cholesterol in isopropanol (1 mg/mL) and egg suspension containing 0.1g yolk were separately incubated with 1mL of cholesterol reductase enriched extract and 1mM NADH for 0, 75min or 24h at 37°C, and residual cholesterol was determined.

One hundred and twenty test-tubes were randomly assigned treatments. Treatments were extract or no extract, incubation time of 0, 75min or 24h and

0.5mL yolk suspension (0.2g/mL) or 0.5mL cholesterol in isopropanol (1mg/mL). Treatments (n=10) were incubated at 37°C and cholesterol was determined at the end of each incubation period. Test-tubes were assayed for cholesterol in numerical order. The arrangement of the test-tubes in the incubator was fully randomised.

The experiment was conducted as a completely randomised design (Snedecor *et al.*, 1967), and data was analysed as a substrate by (enzyme by incubation time, plus control) factorial.

2.3.2 Cholesterol Oxidase

Cholesterol oxidases (COD) derived from Ss, Bs, Ne and Pf were purchased from Sigma Chemical Company, St. Louis, Mo as lyophilised powders. Cholesterol oxidase from *Nocardia erythropolis* was reconstituted in 0.1M pH5 potassium phosphate buffer and the others in the same type of buffer but at pH7. These pHs have been reported as being the most suitable at which to store these enzymes without appreciable loss of activity (Smith and Brooks, 1974).

Since the incubation pH may have a strong effect on the enzymatic activity, the degradation capacity of each enzyme was measured at pH7, a pH at or close to their optimum physiological pH based on available literature. Cheillan *et al.* (1989) observed the activity of Pf COD remaining constant between pH6.5 and 8.5. Uwajima *et al.* (1974) have shown that the enzyme isolated from *Brevibacterium* had a broad pH optimum in the range of pH6.0 to 8.5 and Noma and Nakayama

(1976) observed a pH optimum of 7.0 for Ne and Bs COD, and pH7.5 for the enzyme from *Streptomyces.*

For COD activity, one enzyme unit (1U) is defined as the amount of cholesterol oxidase that will promote the conversion of 1µmole of cholesterol to cholest-4-en-3-one per minute (Figure 2.5) under the prescribed test conditions (Cheillan *et al.*, 1989).

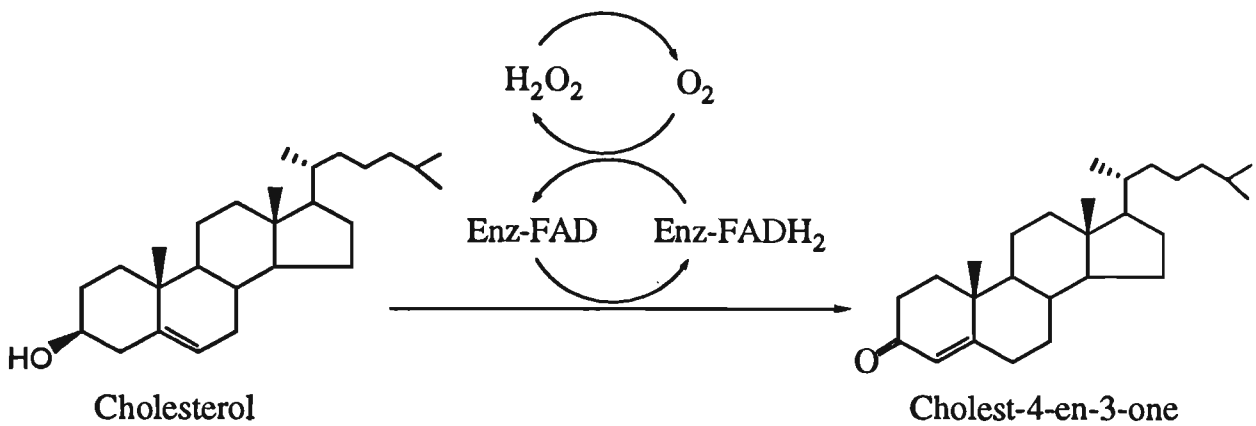


Figure 2.5 The oxidation of cholesterol to cholestenone catalysed by cholesterol oxidase (Cheillan *et al.*, 1989).

Cholesterol Degrading Capacity of Cholesterol Oxidase

Effect of Time and Temperature

The effect of incubation time on the cholesterol degrading capacity of each enzyme was initially investigated. Yolk suspensions containing 0.1g yolk (approximately 3.9µmole cholesterol) were added to tightly capped (teflon-lined) 10mL test tubes and incubated, in triplicate, with 3.9U of each enzyme at 4, 25 and 37°C. Residual cholesterol was determined after 3, 6, 9, 12, 24, 48 and 72h by gas chromatography. The experiment was not replicated. Results of this investigation

were used to select 12h as a suitable time for a more detailed study of incubation temperature.

To four of seven test-tubes containing 0.1g yolk in buffer suspension, 3.9U of cholesterol oxidase enzyme from each of four types (Ne, Ss, Bs and Pf) was added. The other three test-tubes contained no enzyme and were treated as controls. These treatments were randomly positioned into test-tube racks, and the racks were randomly assigned to incubators at each of the temperatures, 5, 15, 25, 37, 45 and 60°C. Incubation time was 12h.

The experiment was replicated on three different days and described as a split-plot type design (Snedecor *et al.*, 1967). Days were considered as blocks, incubators as plots and test-tubes containing yolks as the sub-plots. Temperature was the main plot treatment and the enzymes (including controls) were the sub-plot treatments. The main temperature effect, and the temperature by enzyme type interaction, were divided into orthogonal polynomial components up to cubic order to determine if there were linear and quadratic trends.

The data was analysed as a temperature by enzyme type factorial with the control being considered in the analysis as an enzyme type. Pairs of means were compared using Fisher's least significant difference (LSD) at the 5% level (Snedecor *et al.*, 1967).

Effect of Concentration

Yolk suspensions containing 0.1g yolk cholesterol were incubated with 0, 0.5, 1, 2, 4 and 8U enzyme/µmole egg cholesterol for 2h at 45°C and residual cholesterol was determined. The analysis was replicated on three days, with each day

considered as a block (Snedecor *et al.*, 1967). Within each block there was one test-tube sample of each enzyme type by concentration combination plus two control samples (without enzyme). The arrangement of the test-tubes placed in the incubator was fully randomised. The data was analysed as an enzyme type by concentration factorial plus an external non-enzyme control treatment, with blocking for time. Within the factorial the concentration effects and concentration by enzyme type interaction were divided into orthogonal polynomial components up to cubic order to determine if there were linear and quadratic trends. Pairs of means were compared using Fisher's least significant difference (LSD) at the 5% level (Snedecor *et al.*, 1967).

Thin Layer Chromatography

In order to detect cholesterol oxidation products, thin layer chromatography (TLC) was performed with pre-coated Silica Gel $60F_{254}$ plates (Merck). Incubation products were separated with a mixed solvent system [chloroform:diethyl ether (60:40)] at room temperature and spots were made visible by spraying with 2', 7'-dichlorofluorescein and viewed under ultra-violet light (254nm). Standards of cholesterol and possible oxidation byproducts (5-cholesten-3-one, 4-cholesten-3-one, 25-hydroxycholesterol and 7-ketocholesterol) were also spotted.

Gas Chromatography-Mass Spectroscopy (GC-MS)

The identification of by-products was confirmed using gas chromatographic retention times in conjunction with their mass spectra. Incubation products were extracted twice with diethyl ether (2mL and 1mL), the extracts evaporated to

dryness and the residues dissolved in chloroform (500μL). Samples (1μL) were then taken for GC-MS using a Hewlett Packard 5890 gas chromatograph fitted with a Hewlett Packard 5971A Mass Selective Detector (MSD). The column and chromatographic conditions were the same as for cholesterol determination.

2.4 Microbiological Treatment of Egg Yolk and Milk Cholesterol

Solvents

- Isopropanol anhydrous, Sigma Chemical Co.

Media

- Liquid minimal medium (pH7) contained (g/L):
  ammonium nitrate (NH_4NO_3), 1.0
  di-potassium hydrogen orthophosphate (K_2HPO_4), 0.25
  magnesium sulphate ($MgSO_4.7H_2O$), 0.001
  ferrous sulphate hepta-hydrate ($FeSO_4.7H_2O$), 0.001
  yeast extract, Oxoid L21, 5.0
- Minimal medium agar was prepared from liquid minimal medium containing 1.5% agar, Oxoid L13.
- Neutralised bacteriological peptone, Oxoid L34: 0.1% in distilled water.
- Nutrient agar, Oxoid CM3 containing 0.4% yeast extract, Oxoid L21.
- Nutrient broth, Oxoid CM1 containing 0.4% yeast extract, Oxoid L21.
- Plate count agar, Oxoid CM 325.

Cultures and Media

Cholesterol degrading strains of *Rhodococcus* species were obtained from the Japan Collection of Microorganisms (JCM) as lyophilised preparations. The strains studied were, *Rhodococcus equi* No. 23 (JCM 6819) isolated from butter, *Rhodococcus erythropolis* No. 3 (JCM 6823) isolated from pork fat, *Rhodococcus species* No. 13 (JCM 6830) isolated from chicken fat, and *Rhodococcus species* No.15 (JCM 6831) isolated from chicken fat, as described by Aihara *et al.* (1986).

Stock cultures were prepared by growth on nutrient agar and re-suspension of cells in Nutrient Broth (Oxoid Australia) containing glycerol (15%w/v) and maintained at -20°C. Routine sub-culture was performed in liquid minimal medium at 30°C for 2d.

Growth Curves for Microorganisms

A growth curve was established for each microorganism, so that cells could be harvested for cholesterol degradation studies at the desired stage of growth.

Stock culture (250µL) was inoculated into 10mL nutrient broth and incubated at 30°C with mechanical shaking (220cycles/min). Turbidity readings at 530nm were taken at various time intervals until the stationary phase of the growth cycle was reached.

Detection of COD Activity

Level of Isopropanol Tolerance by Rhodococcus sp.

To measure the cholesterol degrading ability of the microorganisms studied it was necessary to determine cell viability at varying strengths of isopropanol necessary to dissolve pure cholesterol in liquid minimal medium.

Microorganisms (10^6 cells) were inoculated into liquid minimal medium (1mL) containing varying levels of isopropanol: 5, 10, 20 and 50%. Liquid minimal medium provided a minimal requirement of minerals and salts for growth by each culture. Media solutions were incubated at 30°C with mechanical shaking for 24h. Media solutions containing 0% isopropanol were also included and used as controls.

Detection of COD Activity on Agar Medium

Stock culture (250µl) was grown to mid log phase in 10mL Nutrient Broth containing 0.4% yeast extract at 30°C, using an orbital shaker set at 220 cycles/min. Aliquots (1mL) of cell cultures were further diluted in minimal medium broth (100mL).

The four strains of *Rhodococcus* species were inoculated (10µL) on minimal agar plates with and without added cholesterol at a level of 0.4%, and incubated at 37°C for 7d. Cholesterol was dissolved in a 1mL solution of 5% isopropanol in minimal medium prior to addition to minimal medium agar. *Rhodococcus equi* No. 23 was

also inoculated on minimal medium agar containing egg yolk at a level of 33% (equivalent to 0.4% egg cholesterol). Cultures were examined for a halo of cholesterol degradation.

2.4.1 Cholesterol Degradation in Milk

UHT milk sterilised at ultra high temperatures was aseptically diluted, 1 in 2, 1 in 4 and 1 in 8 in liquid minimal medium. Since the cholesterol level in the undiluted milk was 1.42 mg/mL, the diluted cholesterol levels were 0.71, 0.35 and 0.17 mg/mL, respectively.

Undiluted milk and milk dilutions (1mL) were inoculated with cells of *R. equi* No. 23 (10^4cfu) and incubated at 37°C using an orbital shaker incubator set at 220cycles/min. Cholesterol was determined at day 7. Controls incubated for 7d and non-incubated controls were also included. The analysis was replicated five times on the same day. The data was analysed using a two-tailed Student's t-test for the comparison of the means of two independent samples (df=8). Cholesterol degradation was expressed as % reduction.

Milk diluted 8 times (10mL) was inoculated with cells of *R.equi* No. 23 (10^7cfu) previously induced (10µL/mL) in the presence of milk cholesterol (0.19mg/mL) at 37°C for 7d to produce enzymes necessary for the cholesterol degradation. Cultures were incubated in sterile wide-mouthed glass bottles with screw caps for 7d at 37°C using an orbital incubator shaker set at 220cycles/min. Milk was also inoculated with non-induced cells and incubated as described earlier. A sample

(1mL) was aseptically withdrawn at 0 3, 5 and 7d to measure cholesterol. Incubated and non-incubated controls without cholesterol were also included.

The analysis was not replicated. Residual cholesterol was expressed as % reduction.

2.4.2 Cholesterol Degradation in Egg Yolk

Egg yolk, prepared by removing yolk from raw eggs under sterile conditions, was aseptically added to minimal medium (10mL) at varying levels of approximately 0.5, 0.27 and 0.16g. The corresponding cholesterol levels were 0.57, 0.32 and 0.19 mg/mL, respectively.

Yolk sample (10mL) was inoculated with non-induced and induced cells of *R. equi* No. 23 (previously grown in 0.19 mg/mL yolk cholesterol) as described above for the milk test system. Samples (1mL) were aseptically removed at 0, 3, 5 and 7d for analysis of cholesterol. Incubated and non incubated controls were also included. Residual cholesterol was expressed as % reduction.

2.4.3 Cholesterol Degradation Products

The halo section on the plate of *R. equi* No. 23 grown on yolk-containing medium was removed and the cholesterol extracted with diethyl ether and quantitatively analysed by gas chromatography. The diethyl ether extract was also analysed for possible degradation products by gas chromatography-mass spectrometry and thin-layer chromatography. An investigation of the degradation products present in incubated

egg yolk cultures was also performed using thin-layer chromatography and gas chromatography-mass spectrometry.

CHAPTER 3

Optimisation of Cholesterol Assay

3.1 Introduction

There have been numerous methods reported for assaying cholesterol in foods. The classical methods for the determination of dietary cholesterol are based on the Liebermann-Burchard colour reaction or the Zlatkis *et al.* (1953) procedure (Ulberth and Reich, 1992). Other methods include gravimetry, titrimetry, thin-layer chromatography and radioassay (Beyer *et al.*, 1989b; Horvath, 1966). A commonly used method is the enzymatic determination of cholesterol using cholesterol oxidase (Masoom and Townsend, 1985; Karkalas *et al.*, 1982; Kushiro *et al.*, 1982), that usually involves an organic extraction (Beyer *et al.*, 1989b; Warren *et al.*, 1988). A more recent process describes the use of a surface adsorbent which selectively adsorbs sterols from fluid mixtures, such as egg yolk (Klemann and Finley, 1991).

Some of these methods, however, are not strictly specific for cholesterol and have overestimated the true content due to interfering non-cholesterol substances found in various foods (Beyer and Jensen, 1989b; Swift, 1984; Kushiro *et al.*, 1982; Heftmann and Hunter, 1979; Weiss *et al.*, 1964). Chromagens of non-cholesterol substances developed by colorimetric methods are often similar to that developed using cholesterol (Beyer and Jensen, 1989b; Swift, 1984; Kushiro *et al.*, 1982), and sterols closely related structurally to cholesterol react almost as well as cholesterol with the cholesterol oxidase enzyme (Swift, 1984; Kushiro *et al.*, 1982).

The higher values obtained by the colorimetric methods on unsaponifiable yolk extract have been suggested to be due to interference from polyunsaturated fats

(Weiss *et al.*, 1964; Heftmann and Hunter, 1979). The cholesterol content of eggs laid by hens fed 30% safflower oil or linseed oil was overestimated by 36% and 42% respectively, when the direct Zlatkis *et al.* (1953) method for measuring cholesterol was used. For unsaponifiable yolk extracts cholesterol increased by 46% and by as much as 26% in saponifiable extracts (Beyer and Jensen, 1989b). Overestimation of the cholesterol content of eggs as a result of data obtained primarily by colorimetric methods (Beyer and Jensen, 1989b) may lead to errors in determining the effect of egg consumption.

Enzymatic methods that use cholesterol oxidase with a high specificity for 3ß-hydroxy sterol may also in some foods overestimate the cholesterol content. For example, sterols with structures very similar to that of cholesterol coexist in shellfish, thus precise cholesterol measurements in shellfish are difficult (Swift, 1984; Kushiro *et al.*, 1982).

It follows therefore, that methods based on the separation of the cholesterol fraction from interfering chromogens and sterols prior to detection are recommended (Al-Hasani *et al.*, 1990; Beyer and Jensen, 1989b; Heftmann and Hunter, 1979). As a result, chromatographic techniques are now preferred for dietary cholesterol analysis (Ulberth and Reich, 1992; Heftmann and Hunter, 1979).

The procedure of heating the sample with a solution of alkali in alcohol is widely used for separating sterols from total lipid extracts (Tsai *et al.*, 1980; Kou and Holmes, 1985; Maerker and Unruh, 1986; Ramesh *et al.*, 1979). Because the treatment results in complete hydrolysis of saponifiable lipid to yield products

which are soluble in aqueous alkali, sterols and any other non-saponifiable lipids are therefore readily separable by extraction with an organic solvent (Martin *et al.*, 1963; Naber and Biggert, 1985; Sugano and Watanabe, 1961).

An HPLC method used by Beyer *et al.* (1989) showed the cholesterol content of eggs to be much lower than previous measurements and provided a more accurate assessment of the true cholesterol intake associated with egg consumption.

Therefore, in complex biological extracts that contain not only a large excess of other lipids, but also closely related cholesterol analogues, ie., for foods known to contain a mixture of animal fats and vegetable oils, gas chromatography or high performance liquid chromatography methods of analysis are recommended (Beyer *et al.*, 1989; Brown, 1987; Ritchie and Jee, 1985; Karkalas *et al.*, 1982; Tu *et al.*, 1970). Gas chromatography systems, however, have a faster recycle time than liquid chromatography systems, and the potential for more rapid analysis when an automated device is used (Beyer *et al.*, 1989).

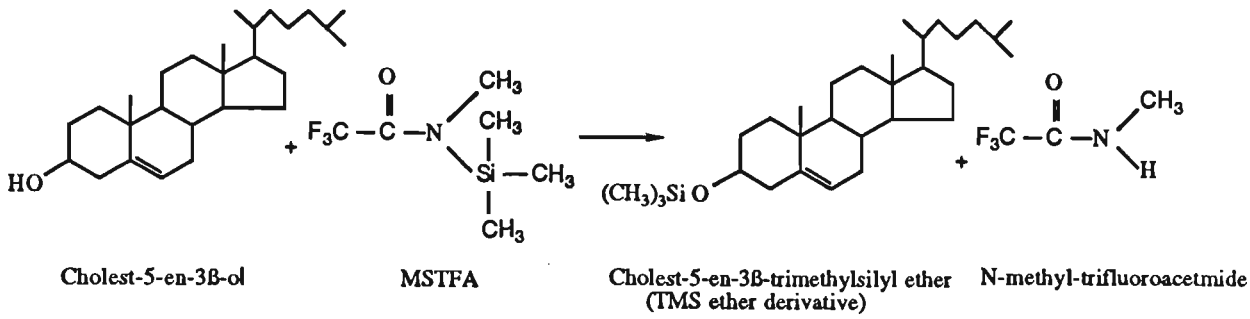
The internal standard procedure is widely accepted as an accurate method for quantitative analysis by gas chromatography (Kovacs, 1990; Bitman and Wood, 1980; Chicoye *et al.*, 1968; Korahani *et al.*, 1981; Morgan and Armstrong, 1989). The method involves the addition of an accurately known quantity of a substance (the internal standard) which is not present in the original mixture. After gas chromatography, the area of the internal standard peak is compared with the area of the peak whose concentration is to be determined. An additional gas chromatography run of a mixture containing known amounts of both the analyte

and the internal standard is normally required to correct for differences in flame ionisation detector response between the two compounds.

Because the basis of this procedure is the ratio of two chromatographic peak areas, the determination is independent of the amount of sample injected onto the gas chromatography column.

Steroids with a free hydroxyl group usually show evidence of adsorption or dehydration when chromatographed under conditions involving relatively high temperatures (280°C to 320°C) (Jennings, 1980). By reaction with a suitable silylating reagent, however, a compound with a free hydroxyl group can be converted to a trimethylsilyl (TMS) ether derivative (Kuksis *et al.*, 1969; Gleispach, 1974). Silylation of the compound reduces loss by adsorption, improves chromatographic efficiency with resultant enhancement of resolution, and yields symmetrical Gaussian-shaped peaks (Luukkainen *et al.*, 1961).

In a non-catalysed silylation reaction a trimethylsilyl (TMS) group is transferred from a donor molecule (silylating reagent) to a receptor molecule by a reaction mechanism which is analogous to that of acyl transfer. The TMS group is usually attached to a nitrogen or oxygen atom in the donor group, and the rate of reaction is affected both by the structure of the silylating reagent and by the structure of the receptor molecule (Chambaz and Horning, 1969). Figure 3.1 shows the silylation of cholesterol using N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) as the TMS donor.



Cholest-5-en-3B-ol MSTFA Cholest-5-en-3B-trimethylsilyl ether (TMS ether derivative) N-methyl-trifluoroacetmide

Figure 3.1 Conversion of cholesterol to the TMS ether derivative (Berezkin, 1983), with N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA)

The character of a molecule is altered from polar and active to non-polar and inert and is generally coupled with an increase in volatility and thermal stability, highly desirable properties for gas chromatography (Chambaz and Horning, 1969).

The rate of derivative formation often depends upon the position of substitution, and consequently vastly different rates of reaction may be observed in the formation of TMS derivatives for functional groups in hindered and relatively unhindered positions (Chambaz and Horning, 1969).

Increased public awareness to the effect of cholesterol on CHD and the relationship of blood cholesterol to the diet has made it necessary to have a rapid, reliable and accurate method to satisfy nutritional labelling demands (Al-Hasani *et al.*, 1990). This study seeks to develop a rapid and efficient procedure that can be used routinely to quantify cholesterol in both egg and other cholesterol containing foods.

3.2 Results and Discussion

The method described by Kovacs (1990) to determine cholesterol in pasta products was modified to measure cholesterol in a wide range of foods. The procedure was significantly improved by employing diethyl ether rather than hexane, the conversion of cholesterol to the trimethylsilyl (TMS) derivative prior to gas chromatography, and the use of either octacosan-1-ol or dihydrocholesterol, rather than cholestane, as an internal standard. The saponification procedure used ensured that total (esterified and unesterified) cholesterol was isolated and therefore gave a high recovery of cholesterol associated with lipoprotein. The smaller volumes of solvent used created limited disposal problems and silylation of the analyte gave a better resolution of chromatographic peaks.

3.2.1 Internal standards

The internal standards, octacosan-1-ol and dihydrocholesterol, were used for different stages of this work. The majority of the work involved in optimising GC conditions was performed using octacosan-1-ol, however the cholesterol analysis of several foods was performed using dihydrocholesterol as an internal standard. Dihydrocholesterol was also used for all of the cholesterol analyses associated with the enzymatic and microbiological cholesterol degradation studies.

Octacosan-1-ol, a long chain alcohol, is isolated from the wax found on green blades of wheat (Merck). Eluting within 2.75min of cholesterol, it did not interfere with other sterols and proved a reliable and accurate internal standard to use.

Unlike octacosan-1-ol, dihydrocholesterol has very similar chromatographic properties to those of cholesterol. It also has similar solubilities to cholesterol in alcohol and diethyl ether. Though dihydrocholesterol and cholesterol are two very closely related substances eluting within 18s of each other, near baseline separation was achieved under the chromatographic conditions used (Figure 3.2).

It was established that interference from other sterols did not occur in GC analysis under the conditions employed since most other sterols eluted at longer retention times than octacosan-1-ol, dihydrocholesterol and cholesterol.

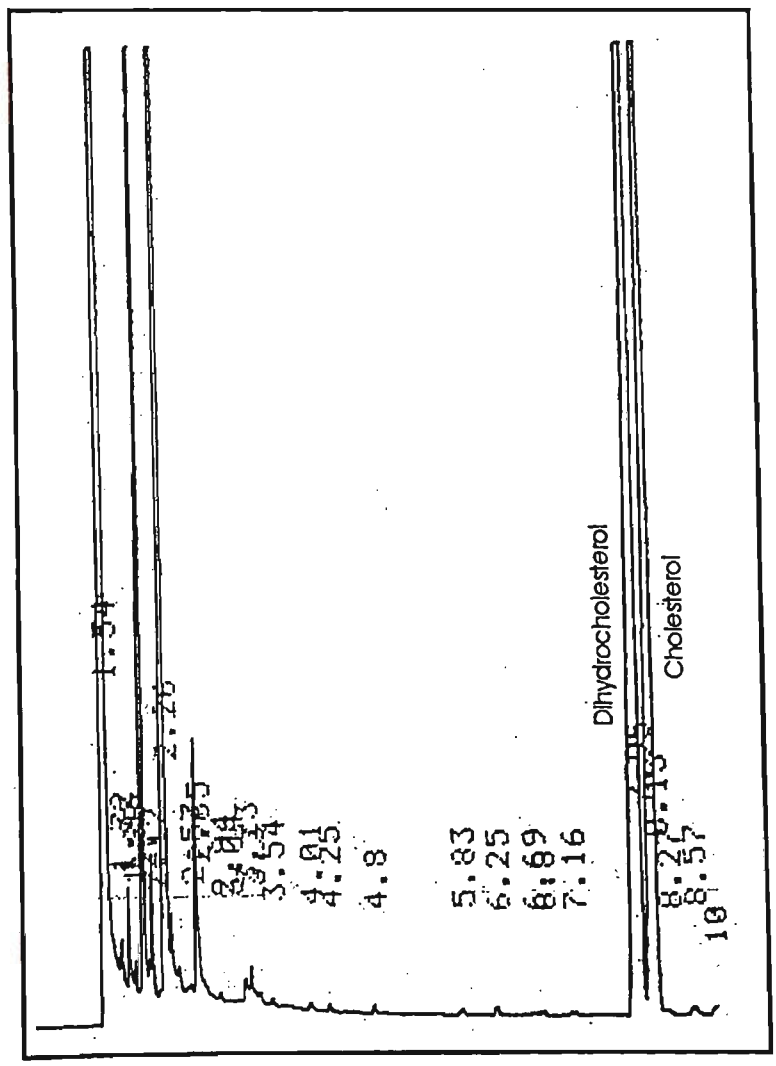


Figure 3.2 Chromatogram showing-near baseline separation achieved between dihydrocholesterol (1) and cholesterol (2) under the chromatographic conditions used.

When the two internal standards were used to measure the cholesterol in egg yolk, the cholesterol values obtained were not significantly different at the 5% level of significance ($p<0.05$) (Table 3.1).

Table 3.1 The analysis of cholesterol in egg yolk using either octacosan-1-ol or dihydrocholesterol as the internal standard.

Internal Standard	Cholesterol (mg/g)*
Dihydrocholesterol	12.04 ± 0.08
Octacosan-1-ol	12.00 ± 0.23

* mean±standard error; n=4.

However, since the chromatographic and solubility properties of dihydrocholesterol are so similar to cholesterol and also because it is relatively inexpensive, readily available and chemically and thermally stable, it is an ideal internal standard for cholesterol analysis and was used for the bulk of the cholesterol analysis reported in this thesis.

Egg yolk was suspended in a buffer medium for all the cholesterol analyses conducted since a greater amount of the cholesterol was extracted than when the yolk was assayed fresh. The cholesterol value (12.32mg/g±0.17) was significantly different ($p<0.05$) to that obtained when the same egg yolk was assayed fresh (12.04mg/g±0.08). Cholesterol appeared to be more accessible to solvent extraction when homogenously suspended in a liquid medium.

3.2.2 Silylation

The hydroxyl groups of cholesterol, octacosan-1-ol and dihydrocholesterol are all sterically unhindered and readily derivatised with N-O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA).

Derivatisation improved the peak shapes of cholesterol, octacosan-1-ol (Figure 3.3) and dihydrocholesterol, and also increased their detector response. Also, because of the similarity in retention time of cholesterol and dihydrocholesterol, the improved peak shape associated with derivatisation significantly enhanced the resolution of these two compounds (Figure 3.2).

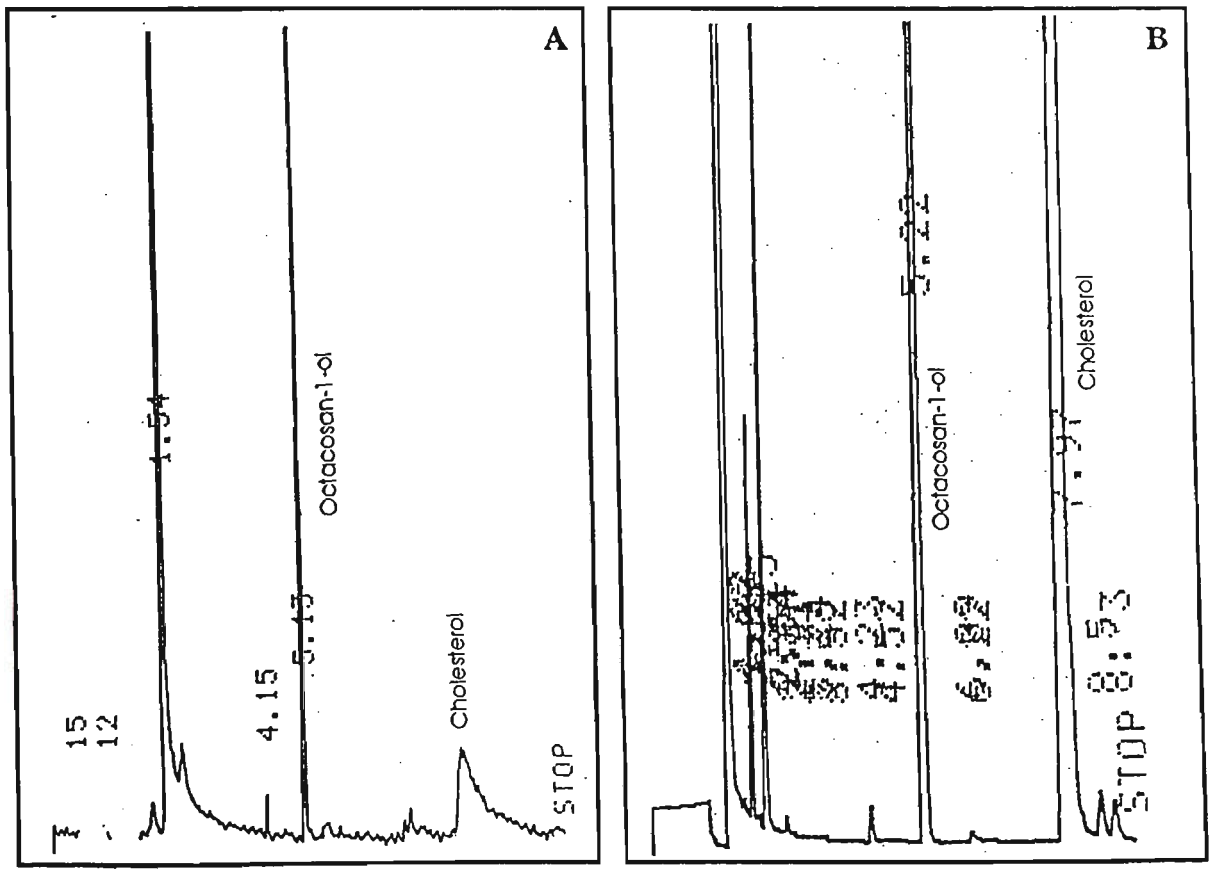


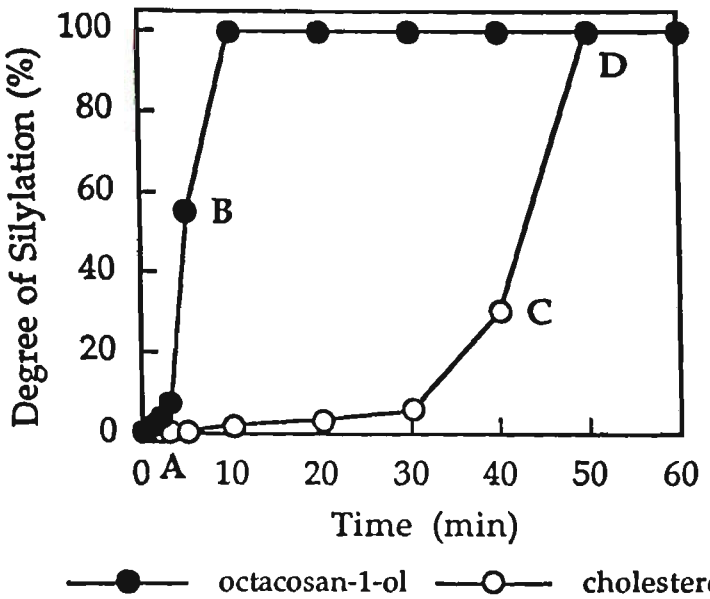
Figure 3.3 Gas chromatograms under identical conditions showing (A) peak shape quality of underivatised octacosan-1-ol and cholesterol, and (B) improved peak shape quality after derivatising with MSTFA.

Silylation Reaction Time Study

To establish appropriate conditions for derivative formation prior to GC, the relative rates of reaction of hydroxyl groups with the two silylating reagents, N-O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), were examined under various reaction conditions. This study indicated that these reactions can be accelerated by heating (Chambaz and Horning, 1969). Silylation was considered complete when none of the unsilylated components of the standard mixture could be detected in the chromatogram.

The ease of derivative formation varied greatly between cholesterol and octacosan-1-ol. Although BSTFA has the ability to release two TMS groups, rapid silylation at room temperature was not observed. Complete silylation of the reaction mixture required up to 50min at 100°C (Figure 3.4) compared to 15min with MSTFA. Excess MSTFA (100µl) gave complete silylation of the standard mixture at room temperature, in less than one minute, but for an egg yolk extract sample, complete silylation required 15min at 100°C.

Both reagents form silyl derivatives with the sterically non-hindered hydroxyl groups, but the high silyl-donor effectiveness of MSTFA made it an excellent reagent for the silylation of cholesterol and the internal standards.



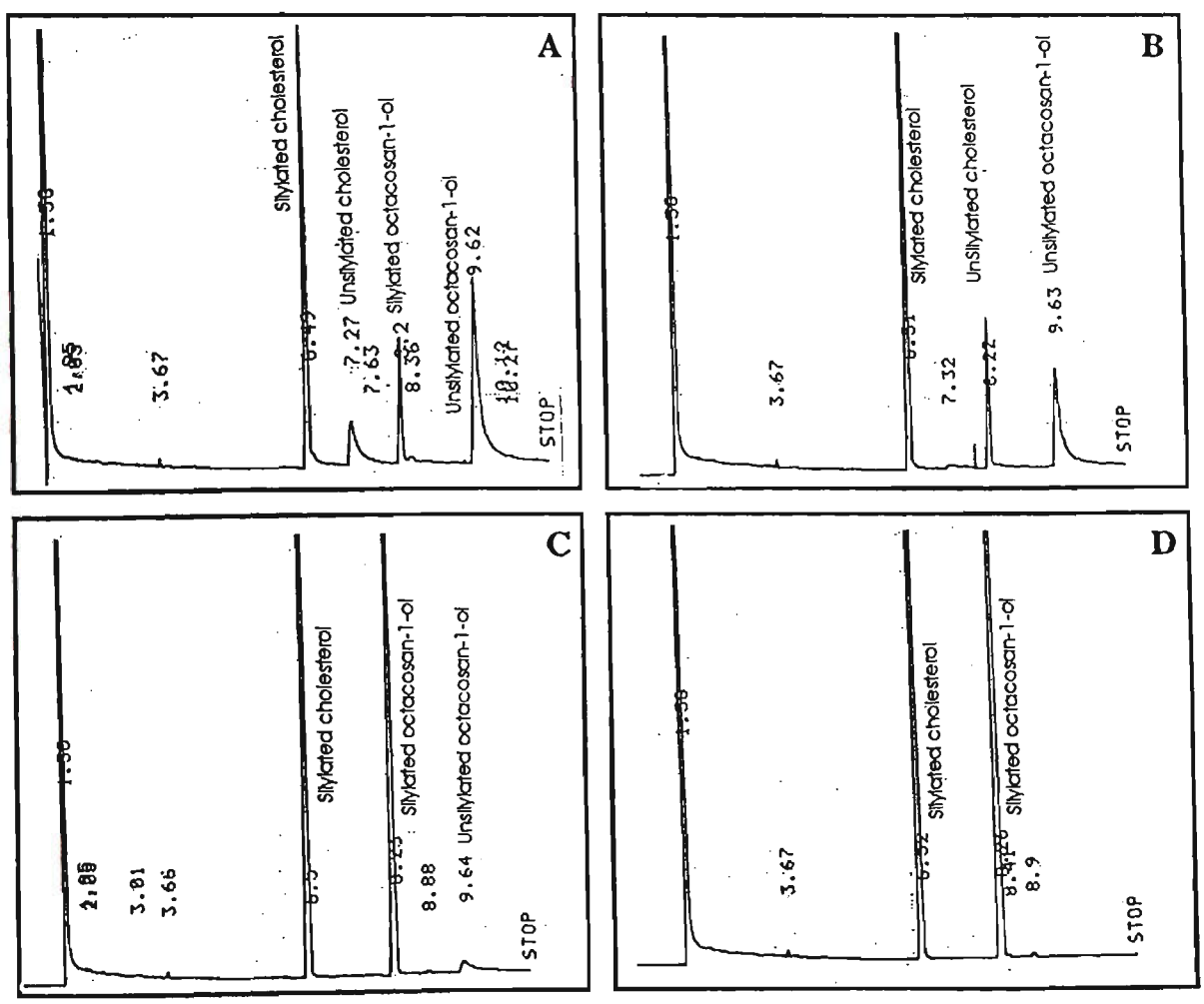


Figure 3.4 The rate of derivative formation in a mixture containing equal amounts of octacosan-1-ol and cholesterol using BSTFA at 100°C, and gas chromatography traces of the reaction mixtures after (A) 2min, (B) 5min, (C) 40min and (D) 50min.

3.2.3 Position of Capillary Column in Varian Gas Chromatograph Injector

Peak quality as indicated by both Gaussian symmetry and magnitude of area was found to be influenced by the position of the inlet end of the capillary column in the glass-lined metal tube leading down from the injector into the gas chromatograph oven. The peak quality was judged optimal when the peak height ratio and peak area ratio obtained from the cholesterol/octacosan-1-ol standard were both nearly unity; this occurred when approximately 8cm of the capillary column was inserted into the glass liner of the injector port (Figure 3.5).

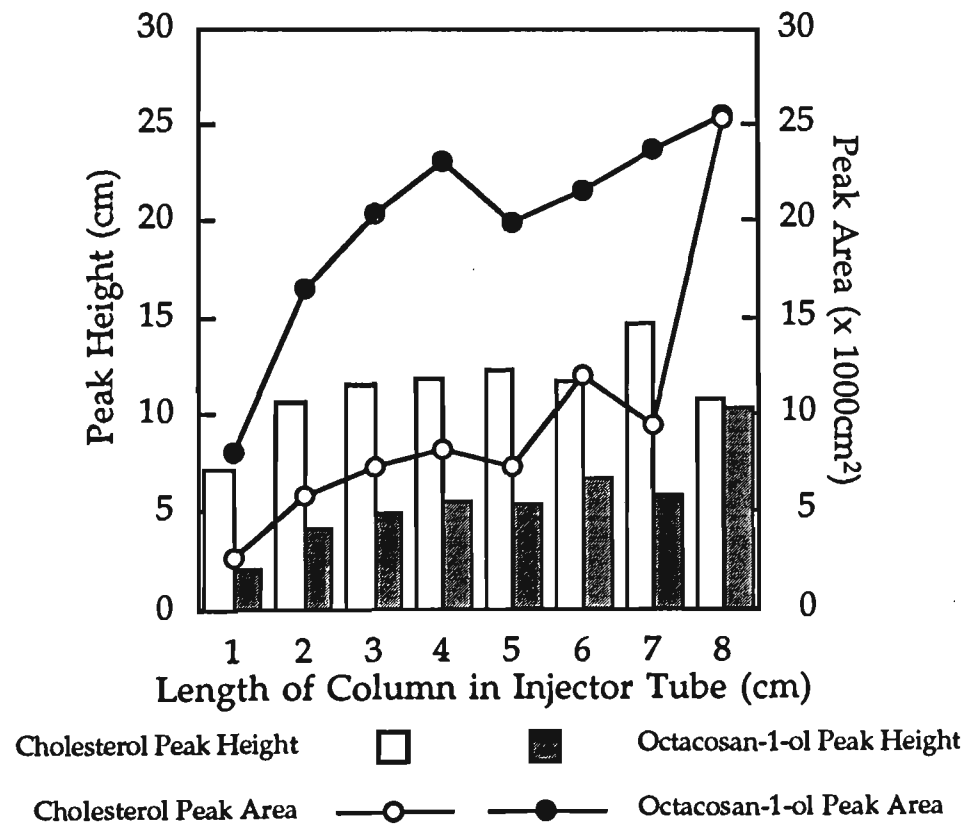


Figure 3.5 The effect on peak quality by varying the length of capillary column inserted into the injector tube of a gas chromatograph: measured by the ratio of peak area between cholesterol and octacosan-1-ol and the peak height of the same two compounds.

When the length of the column inserted was less than 8cm or greater than 8cm, bad tailing on the trailing edge of peaks, and significant differences in peak heights was observed (Figure 3.5).

3.2.4 Relative Response Factors

The response of a detector is the quantity of a signal generated by a given amount of the sample. The general rule is that equal amounts of components with similar functional groups give equal responses, however, correction factors are usually necessary when different functional groups are involved.

The relative response factor, measured as the peak area of the internal standard relative to cholesterol, was 1.064 (n=8) and 1.022 (n=4) for dihydrocholesterol and octacosan-1-ol, respectively. The respective relative response factor was then incorporated as a correction factor into all cholesterol determinations.

3.2.5 Determination of Cholesterol

The identification of cholesterol and dihydrocholesterol in gas chromatography traces was facilitated by comparison of retention times with those of known standards chromatographed under identical conditions. Quantification of cholesterol was achieved by comparison of the areas of the cholesterol and dihydrocholesterol peaks and the application of a small factor to recognise a slight difference in the response of each of these compounds in the flame ionisation detector.

3.2.6 Reproducibility and Recovery

Reproducibility data collected for egg yolk for each assay procedure yielded markedly different results; the modified procedure gave a higher cholesterol value, along with a lower coefficient of variation, than the Kovacs procedure (Table 3.2).

Table 3.2 Comparisons of extraction by the modified and original procedures in egg yolk.

Procedure	Reproducibility		% Recovery
	mean±s.e.	C. V.	mean±s.e.
	mg cholesterol/g yolk		
Modified [1]	16.10±0.06	1.1%	103.1±1.5
Original [2]	10.07±0.11	3.1%	71.7±2.0

C. V.=coefficient of variation
[1] n=8
[2] n=7

The amount of cholesterol recovered by the modified procedure, when 10mg/g of cholesterol was added to a series of egg yolk samples, was 10.31±0.15mg/g, however, only 7.17±0.20mg/g was recovered by the Kovacs procedure. The Kovacs procedure was, therefore, extracting only about two thirds of the available cholesterol as shown by both the reproducibility and recovery data.

For uncooked noodles, a lower coefficient of variation and a higher % recovery was obtained with the modified procedure, while the original procedure gave a higher coefficient of variation and a lower recovery. The difference ± s.e. for the

recoveries using the original and modified procedures were 2.52±0.04mg/g and 1.96±0.08mg/g, respectively.

3.2.7 The Determination of Cholesterol in Multi-component Foods

When comparing the method of Kovacs (1990) with a laboratory kit used for the enzymatic determination of cholesterol, Karkalas *et al.* (1982) found very good agreement between the test materials analysed. All of the test materials assayed in the Karkalas *et al.* investigation, however, contained less than 2mg cholesterol/g sample. The reproducibility and recovery for cholesterol extracted from pasta samples with low cholesterol (1.52mg/g) by either method in this study and those reported by Karkalas *et al.* (1982), are in contrast with those obtained from egg yolk with high cholesterol (16.10mg/g). It is suggested that the accuracy of the Kovacs method is affected by the level of cholesterol in each type of food.

To assess the suitability of the modified gas chromatographic method for measuring cholesterol in foods other than egg, the cholesterol content of several foods available in Melbourne markets was investigated (Table 3.3). The chromatograms for these foods gave excellent baseline separation without interferences and triplicate samples of the same food type indicated repeatability between assays.

The Italian egg spaghetti had a cholesterol content in the range 0.15-0.79mg/g while egg noodles had 0.93-1.42mg/g. The high level of cholesterol found in egg noodles was presumably due to their being prepared with egg yolk, while the Italian pasta was made with whole egg. Some egg rolls had very high cholesterol

contents (1.83-3.84mg/g). In meat-based products, ham products had a cholesterol level similar to pork cakes or pork loafs (0.63-0.71mg/g compared with 0.70-0.86mg/g, respectively). A similar cholesterol level was also found among fish balls and cheddar cheese (0.99mg/g and 1.01mg/g, respectively). Chicken liver, chicken paste and prawn had the highest cholesterol contents among the products investigated (Table 3.3).

Table 3.3 Cholesterol (mg/g±s.e.*) content of various foods.

MEAT PRODUCT	mg/g±s.e.	EGG PRODUCT	mg/g±s.e.
Fish Balls	0.99±0.04	TAK ON ASIAN FOOD Pure Egg Noodle	1.42±0.05
Pork Loaf	0.86±0.004	MAI HONG Egg Noodle	1.65±0.08
Pork Cake	0.70±0.03	INDO CHINA Chow Mein	0.93±0.03
DON SMALLGOODS DELI SUPREME Premium Unsmoked Ham	0.63±0.01	PHOENIX Egg Rolls	1.83±0.15
CASTLE Strasberg	0.67±0.02	KHONG GUAN Egg Rolls	3.84±0.24
HERITAGE PATE Chicken Liver with Brandy	2.75±0.2	VETTA Egg Spaghetti	0.23±0.01
REGAL PATE Grand Manier	2.63±0.19	SAN REMO Vegeroni Egg Spaghetti	0.15±0.01
Bacon Meat	0.82±0.04	GUZZIS Tagliolini	0.79±0.01
Bacon Fat	1.29±0.03	Prawn	2.49±0.09
Chicken Fillet	0.74±0.05		
Chicken Liver	4.25±0.04	OTHER	mg/g±s.e.
Lean Topside	0.76±0.02	MAI HONG Won Ton Skin	0.14±0.04
Beef Loin	0.83±0.02	Cheddar Cheese	1.01±0.08

* n=3 for all foods analysed

The method demonstrated a capacity to extract cholesterol in both non-egg and egg-containing foods with different and complex matrixes, and the cholesterol level extracted did not appear to be affected by the level of cholesterol with as little as 0.23mg/g cholesterol and up to 16mg/g for egg yolk extracted.

CHAPTER 4

Degradation of Egg Yolk Cholesterol using Purified Enzymes

4.1 Introduction

Enzymes are proteins containing the same amino acids found in other proteins, and like other proteins have a three-dimensional structure that represents the conformation of least energy content. Enzymes function as catalysts, ie., they speed up chemical reactions which would otherwise proceed slowly, or not at all (Dziezak, 1991). This is the basis for enzyme function; the spatial arrangement of amino, carboxyl, and other bonding groups increases catalytic effectiveness by orders of magnitude (McGilvery, 1979). Reaction times for industrial enzymes vary from a few minutes to several days (Dziezak, 1991).

4.1.1 Enzymatic Reactions

An enzymatic reaction proceeds through at least three stages (McGilvery, 1979):

1. the formation of a complex between enzyme and substrate (ES),
2. the conversion of this complex to an enzyme-intermediate complex (EI), and
3. further conversion to a complex between enzyme (E) and product (P) that can dissociate, releasing both the product and the regenerated active enzyme:

$$E + S \rightleftharpoons ES \rightleftharpoons EI \rightleftharpoons EP \rightleftharpoons E + P$$

Enzymes owe their peculiar catalytic effectiveness to a combination of specific binding and the presence of catalytic groups. For example, many amino acid side chains are capable of donating or accepting protons so as to act as general acid or general base catalysts. Other groups, such as hydroxyl or amino groups, may act as nucleophiles (donate electrons to bond to other groups with vacant orbitals)

(McGilvery, 1979) and moieties other than amino acids in the protein chains of enzymes, such as vitamins and metal ions, may also be involved in the catalytic process (Williams, 1969).

The binding and catalytic groups of the enzyme combine with the substrate in such a way that the intermediate-enzyme complex has a lower energy content than the complexes of either the substrate or the product, with the enzyme. The activation energy for formation of the intermediate-enzyme complex is therefore much lower than the activation energy for the reaction in the absence of enzyme (McGilvery, 1979).

4.1.2 Enzyme Specificity

Enzymes are highly specific in binding particular substrates, because the amino acid residues in the binding site closely fit only a few compounds, in some cases perhaps only one (McGilvery, 1979). Therefore, changes in the activity of particular enzymes will affect only a few compounds, an important feature of biological control (McGilvery, 1979).

The specificity of enzyme reactions is a very significant factor when evaluating enzymatic processes versus conventional chemical processes. Because of the specificity of enzymes, large amounts of compounds can be broken down or synthesised with minimum formation of byproducts. Enzymes also permit reactions to take place under mild operating conditions of temperature, pressure and pH, which generally means reduced energy costs. Further, low usage levels make the use of enzymes economical as well as practical (Dziezak, 1991).

4.2 Conversion of Cholesterol in Food to Coprostanol by Cholesterol Reductase

4.2.1 Introduction

Cholesterol reductase (CR) is an enzyme that transforms cholesterol into coprostanol, an innocuous compound that is believed to remain inert as it passes through the digestive system (Best, 1989). The reductase enzyme was discovered in certain green plant parts and is known to be present in several bacteria that commonly inhabit the digestive tract of animals (Beitz *et al.*, 1990).

A patented process of concentrating cholesterol reductase from an homogenate of cucumber plant leaves has been described (Beitz *et al.*, 1990). The process involves homogenising the green plant parts, separating the fibrous materials from the crude homogenate, and concentrating the homogenate to provide a cell-free, cytosolic, cholesterol reductase-enriched preparation, or ultimately a cholesterol reductase purified to homogeneity.

The authors of the patent reported a reduction in cholesterol in food substances after treatment with the CR-enriched preparation, and a cholesterol reduction in animals to be used to provide meat after intravenous administration of a CR-enriched preparation an hour prior to slaughter. The reduction in animals was described as a 50% reduction "most of the time" and a 25% reduction "all of the time" (Beitz *et al.*, 1990). The same researchers stated that the enzyme can also be added to ground preparations of meats.

To reduce cholesterol levels, homogenised milk can be treated directly with the CR-enriched preparation or alternatively passed through an enzyme-supporting inert material. Intact whole eggs can be treated by injecting CR enzyme into the yolk and broken egg mixtures can be treated directly with the CR enzyme (Beitz *et al.*, 1990).

Cholesterol reductase activity has also been measured in *Eubacterium species* Strain HL (Freier *et al.*, 1992). The culture bacteria could be redesigned so that they produce their own cholesterol reductase activity (Best, 1989). For example, by genetically altering the starter cultures the bacteria could produce cholesterol reductase while cheese is aging (Hegenbart, 1989). Potential organisms from which cholesterol-reducing enzyme activities could be transplanted into cheese starter cultures have already been identified, but a redesigned dairy culture has not yet been produced nor is it known how the genes will be expressed (Best, 1989).

4.2.2 Results and Discussion

The patented procedure for extracting CR from cucumber leaves by Beitz *et al.*, (1990) was used to investigate the cholesterol degrading ability of the cholesterol reductase enzyme in an egg yolk system. Unfortunately, although the procedure for extracting the enzymes was followed carefully, the results reported by Beitz *et al.* (1990) could not be repeated.

In the experiment examining the cholesterol degrading capacity of the cholesterol reductase extract with egg yolk suspension and with pure cholesterol, there was no evidence ($p>0.1$) of any extract effect nor any interaction of extract with medium

or incubation time. The mean cholesterol level after treatment with the extract was 0.50mg/g (s.e.=0.682) less than the untreated sample, with a 95% confidence interval of (-0.87, 1.87). These limits correspond to a range extending from a 7% increase to a 15% decrease, respectively.

The cholesterol level in the yolk was much higher than in the pure cholesterol mixture (13.79mg/g vs 10.37mg/g, sed=0.305, $p<<0.001$). There was statistical evidence that cholesterol increased after 24h in the incubator, with or without extract, (mean=11.62mg/g vs 9.13mg/g for external control, sed=0.591), but this is almost certainly an artefact.

Several unsuccessful attempts were made in May, 1991 to contact Akiva Gross, author of "Enzymatic Catalysis in the Production of Novel Food Ingredients" (Food Technology, Jan 1991) in which he made reference to work being conducted with CR at Iowa State University. Faxed inquiries seeking information about the availability of pure CR in the US, and the identity of the relevant CR researchers were not answered. An attempt to contact Akiva Gross by telephone was also unsuccessful. Further attempts to make contact were abandoned.

It may be that cellular release of the cholesterol reductase was not achieved during the homogenisation of the cucumber leaves. Although precautions were taken to keep the homogenised slurry cool during the homogenisation process, the increasing temperature created may have resulted in the denaturation of the enzyme.

Beitz *et al.* (1990) measured the ability of the CR-enriched preparation to degrade cholesterol in a complex food system, such as milk, by treating milk with

cholesterol oxidase, a model enzyme for oxidising cholesterol. The amount of cholesterol degraded by the oxidase enzyme was considered to be the same amount that would be accessible to cholesterol reductase if the milk was treated with the reductase enzyme, and so a measure of reductase degrading ability. On this basis, cholesterol oxidation revealed that up to 80 to 90% of the cholesterol in homogenised, pasteurised whole milk would be accessible to reduction to coprostanol by treatment with cholesterol reductase, and 60 to 80% in raw whole milk.

These results should be treated with suspicion however, since the ability of cholesterol oxidase to catalyse the oxidation of cholesterol in milk seems unlikely to be mirrored by the ability of cholesterol reductase to catalyse the reduction of cholesterol. The two reactions are clearly different and the accessibility of the substrate, as well as the availability of the other reagent (oxidant or reductant), may be quite different in each case.

Indeed, Dehal *et al.* (1988) reported a 7% reduction in cholesterol after treating free cholesterol directly with extracellular cucumber leaf CR for 75min at 35°C. Since this work was performed with free cholesterol, the accessibility of cholesterol in this preparation, would presumable be described as 100%, a cholesterol availability that may not be the case in more complex systems such as ground meat, milk or eggs. The cholesterol reductase activity identified in *Eubacterium species* Strain HL (Freier *et al.*, 1992) was similarly not directly tested in more complex food systems.

Cholesterol reductase extracted from other plant sources such as Alfalfa (*Medico sativa*) and pea (*Pisum sativum*) leaf converted cholesterol to coprostanol at a rate of 23nmole and 2.6nmole/h/mg protein, respectively, at the temperature optimum of 37°C (Dehal *et al.*, 1990, 1991). Though, the cholesterol degrading ability of alfalfa and pea leaf cholesterol reductase was demonstrated, the importance of these very small conversion rates to the consumer seeking to restrict dietary cholesterol, is at best doubtful.

The difficulties that would be encountered in using CR in commercial applications to reduce the cholesterol in foods would include (1) identifying commercially viable sources of the CR enzyme and its associated cofactors, and (2) achieving high enzyme yields.

4.3 Conversion of Cholesterol in Food to Cholestenone by Cholesterol Oxidase

4.3.1 Introduction

Another enzyme, a cholesterol:oxygen oxidoreductase called cholesterol oxidase (COD), has been identified as demonstrating a specificity for cholesterol and it's derivatives (Smith and Brooks, 1974; Moore *et al.*, 1977; Tomioka *et al.*, 1976; Ikawa *et al.*, 1979; Cheetham *et al.*, 1982; Inouye *et al.*, 1982; Uwajima *et al.*, 1974; Aihara *et al.*, 1988; Cheillan *et al.*, 1989).

Preparation and Characterisation of COD

COD is an inducible intracellular enzyme and can be prepared by incubating bacterial cells in a cholesterol medium (Flegg, 1973). Bacterial cells of *Nocardia erythropolis* have been grown in such a medium by Flegg for the production of the enzyme.

The COD produced was separated by sonication of the cholesterol medium to rupture the cells and centrifugation to sediment any residual cell matter and cholesterol. As the bacterial cells contain many other enzymes, especially enzymes acting on cholest-4-ene-3-one, the COD can be purified further by ammonium sulphate fractionation of the cell-free supernatant.

This partially purified COD mixture can then be used to determine cholesterol in serum, for example, based on the steady accumulation of cholest-4-ene-3-one in the oxidation of cholesterol by the COD enzyme.

Although, complete specificity for cholesterol is not exhibited by COD, the enzymatic technique is more specific than colorimetric cholesterol assays. COD enzyme can also be isolated from the broth filtrates of *Streptomyces violascens* (Fakuda *et al.*, 1973; Tomioka *et al.*, 1976), *Brevibacterium sterolicum* (Uwajima *et al.*, 1973, 1974) and *Streptoverticullim cholesterolicum* (Inouye *et al.*, 1982).

COD from *Nocardia species* and *Pseudomonas* were characterised by Richmond (1973) and Cheillan *et al.*(1989) respectively with a view to performing enzymatic determinations of cholesterol.

COD from *Pseudomonas fluorescens* exhibited constant activity over a range of cholesterol concentrations (2.59, 5.18 or 10.36mM) provided that the pH of the medium was maintained between 5.5 and 8.5 (Cheillan *et al.*, 1989). Enzyme that is immobilised must remain stable for a long time in the reaction medium. The effect of this prolonged incubation on the retention activity of COD was studied by Cheillan *et al.* (1973) and the most favourable pH for the conservation of this protein was found to be near the isoelectric point (pH 5 to 5.5). This could be of value especially in treatments aimed at immobilising enzymes.

The bulk of egg cholesterol is found in the low-density lipoprotein (LDL) particle (Gornall and Kuksis, 1971) and a large proportion of the cholesterol in LDL (approx. 92%) is extractable with ether (Evans *et al.*, 1973). This suggests that cholesterol in egg yolk LDL is not involved in strong interactions with macromolecules and is therefore likely to be degraded more easily by COD than cholesterol present in other classes of lipoproteins which cannot be extracted with ether (Evans *et al.*, 1973).

Specificity and Mode of Action of COD

The specificity of COD was demonstrated by comparing the rates of oxidation of several cholesterol analogues with that of cholesterol at the same molarity. COD was specific for 3β sterols and required a double bond in the Δ^5 or Δ^4 position of the sterols (Richmond, 1973). Of a number of cholesterol analogues tested, 5α-cholestan-3β-ol and 5β-cholestan-3β-ol were not oxidised by cholesterol oxidase (Allain *et al.*, 1974).

A list of cholesterol derivatives able to interfere in the enzymatic reaction of COD (Ikawa *et al.*, 1979) were characterised by a hydroxyl function in position β in relation to the cyclic nuclei (Cheillan *et al.*, 1989). Certain microorganisms also contain highly specific oxidases which oxidase one specific hydroxyl group on the third carbon in preference to another (Smith and Brooks, 1977).

In contrast, *Nocardia erythropolis* COD, a soil organism (Turfitt, 1944), was found to act on 5α-cholestan-3β-ol, such as stigmasterols and sitosterol, as well as Δ^5-3β-hydroxysteroids (Smith and Brooks, 1974) found in egg yolk (Tu *et al.*, 1970). Although *Nocardia erythropolis* COD does not show complete specificity for cholesterol it is more specific than present colorimetric cholesterol assays and exhibits comparable precision (Flegg, 1973).

The rate of oxidation of Δ^5-3β-hydroxysteroids was also found to be influenced by side-chain length (Smith and Brooks, 1974). COD from *Nocardia erythropolis* showed a markedly diminished affinity for cholesterol when the side-chain was shortened (Richmond, 1973). In contrast, COD from *Brevibacterium sterolicum* did not show any marked change in activity as the length of the side-chain was altered (Ikawa *et al.*, 1979).

The position and type of side-chain oxygenation also effects cholesterol oxidation (Smith and Brooks, 1974). A hydroxyl group in the 25- or 26-position of the cholesterol side-chain decreases the affinity of the enzyme for a steroid, illustrated by the difference in rates between cholesterol and 26-hydroxycholesterol (Smith and Brooks, 1974). Shortening the side chain at C-17 to just one carbon, or the

complete removal of the side chain, results in a dramatic reduction in the rate of oxidation, though the apparent Km is unaffected (Smith and Brooks, 1977).

COD from *Brevibacterium sterolicum* catalyses the oxidation of steroids having a sterically unhindered C-3β hydroxyl group, but shows weak activity towards steroids with functional groups adjacent to the 3β-hydroxyl group on the steroid nucleus (Ikawa *et al.*, 1979).

Cofactors of Cholesterol Oxidase

Like many oxidation enzymes, COD acts in association with a cofactor which ensures the transfer of electrons and hydrogen atoms from cholesterol to molecular oxygen. The oxidoreduction cofactor has been identified as covalently bound flavin adenine dinucleotide (FAD) (Kenney and Singer, 1979). FAD has also been identified as the flavin moiety of the enzyme from *Brevibacterium sterolicum* ATCC 21387, one mole of which was found per mole of protein (Uwajima *et al.*, 1974).

Although, FAD is covalently bound to the enzyme, addition of exogenous FAD to the buffer solution was found to double COD activity. However, the increase in enzyme activity was not proportional to FAD concentration (Cheillan *et al.*, 1989).

Cholesterol Oxidation Byproducts

The byproduct of cholesterol oxidation has been identified as cholesten-4-en-3-one, by mp, elemental analysis, optical rotation and ultra-violet, infra-red and

nuclear magnetic resonance spectra (Uwajima *et al.*, 1974). Smith and Brooks (1974) have demonstrated that cholesten-5-en-3-one, a structural isomer of cholesten-4-en-3-one, is an intermediate in this process. Cholesterol oxidase thus possesses both oxidase and isomerase activity. Investigations on substrate specificity of isomerase action using 5-cholesten-3-one, pregnen-5-ene-3,20-dione and androst-5-ene-3,17-dione, showed there was no major influence of the C-17 side chain on the rate of isomerisation; however, in the absence of a side-chain, a large increase in the apparent Km was observed.

The cholesterol oxidase reaction of cholesterol to cholestenone has been described in many microorganisms including *Nocardia erythropolis* (Flegg, 1973; Smith and Brooks, 1974) and *Nocardia rhodochrous* (Cheetham *et al.*, 1982).

COD Activity and Cholesterol Assay

The assay of COD activity is based on the conversion of cholesterol to cholestenone which has an absorption maximum of 240nm, owing to the conjugated carbonyl ring group in ring A (Allain *et al.*, 1974; Flegg, 1973). Determination of the byproduct of cholesterol oxidation has been used by many investigators for the assay of cholesterol and the measurement of COD activity, as there is a stable accumulation of the product allowing analysis to be made at equilibrium (Flegg, 1973).

Auto-oxidation byproducts of cholesterol such as 25-hydroxycholesterol and 7-ketocholesterol (Csallany *et al.*, 1989) and cholesta-3,5-diene-7-one (Maerker and Unruh, 1986) have been referred to as toxic (Naber *et al.*, 1983). The 7-

ketocholesterol may not be detected by workers treating cholesterol with the COD enzyme, as the product has long been known to be unstable to hot aqueous alkali (Maerker and Unruh, 1986). Therefore, it would most probably be destroyed in the saponification step in routine sterol extraction. These products, however, are due to the spontaneous oxidation of cholesterol rather than enzymatic oxidation.

All previous enzymatic methods for cholesterol determination have been based on the conversion of cholesterol to cholestenone and hydrogen peroxide, which are produced in equimolar quantities. Hydrogen peroxide, produced during enzymatic reaction and serving as a basis for many electrochemical detection techniques, is an inhibitor of COD. Cholest-4-en-3-one has no effect on the enzymatic reaction (Cheillan *et al.*, 1989).

Noma and Nakayama (1976) after studying the reaction rates at varying concentrations of cholesterol by COD from *Brevibacterium* and *Streptomyces* concluded that both enzymes were suitable for the determinations of cholesterol in hypercholesterolaemic sera.

Potential Application for Cholesterol Degradation

The potential of COD enzyme for reducing cholesterol in liquid egg yolk has been indicated by work performed on milkfat (Xiansheng *et al.*, 1990). Milk is a complicated system, similar in many ways to egg, and was selected as a trial food system to investigate the cholesterol degrading capacity of the enzyme. When commercial milk was treated with COD isolated from *Streptomyces species*, up to 65% of cholesterol in milk was removed after 24h incubation at low temperature

(4-7°C) (Xiansheng *et al.*, 1990). Other researchers have demonstrated the ability of cholesterol oxidase to degrade all of the cholesterol in a buffer system and up to 65% in milk at low temperature (3-7°C), in less than 24h (Uwajima *et al.*, 1974).

The cholesterol degrading activities of cholesterol oxidases originating from four different microorganism genera, *Brevibacterium, Nocardia, Pseudomonas* and *Streptomyces*, in an egg yolk system under different environments was investigated, in order to define the optimal conditions for each enzyme. The enzyme from *Nocardia* is intrinsically membrane bound whereas those from *Streptomyces* and *Brevibacterium* are extracellular cholesterol oxidases (Stehbens, 1990; Cheillan *et al.*, 1982; Smith and Brooks, 1974).

The cholesterol content of treated and untreated egg yolks were compared and the results expressed as either percent cholesterol reduction or mg/g residual cholesterol.

4.3.2 Results and Discussion

Effect of Time and Temperature

The time course of cholesterol degradation resulting from each of four CODs in egg yolk at 37°C, 25°C and 4°C are shown in figures 5.1, 5.2 and 5.3. In each case, the yolk was incubated with enzyme for 72h.

The cholesterol degradation activity of each enzyme at 37°C is presented in figure 5.1.

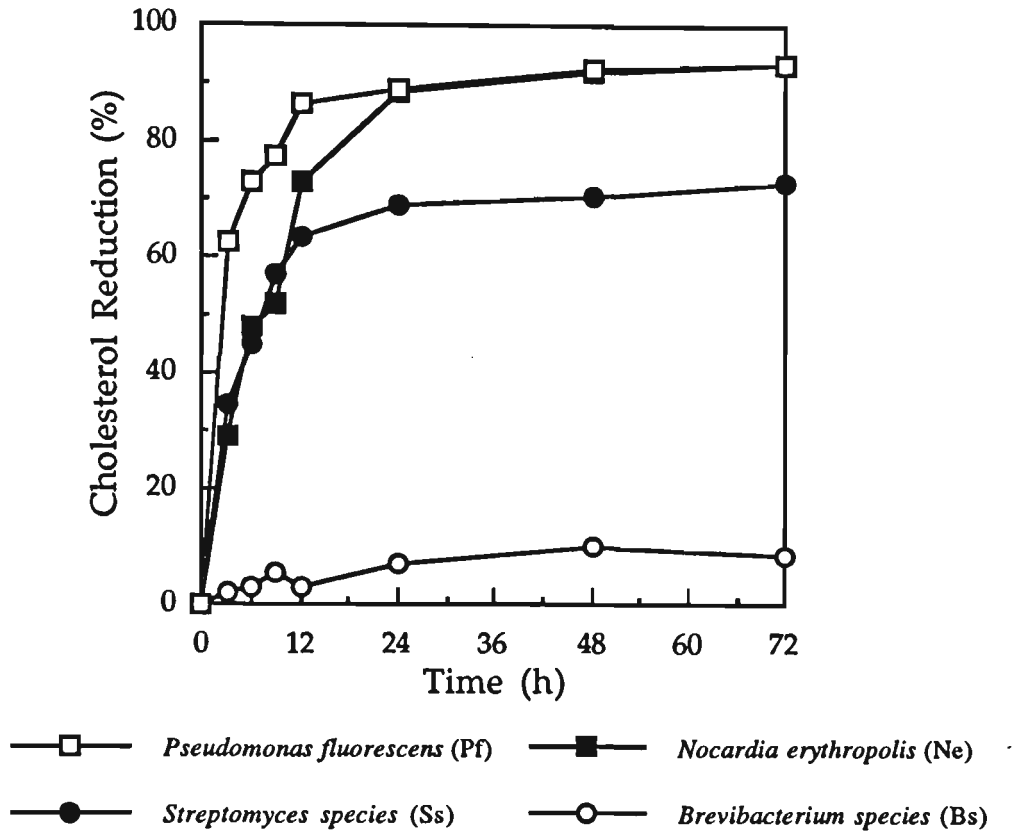


Figure 4.1 Time course of cholesterol degradation in egg yolk at 37°C by the four CODs investigated. Each data point is the average of triplicate samples.

At 37°C, the Bs COD could only degrade about 8.3% of the total cholesterol after 72h of incubation while Ss COD reduced cholesterol by 72.6%. Both Pf COD and Ne COD, respectively, reduced up to 93.4% of the cholesterol.

With the exception of Bs COD, cholesterol degradation at 37°C occurred in two stages. In the first stage, the reaction proceeded rapidly up to approximately 12h. In the second stage, the reaction rate became slower, and after 24h remained almost unchanged.

The performance of the Pf COD at 25°C (figure 4.2) was similar to that at 37°C in terms of reaction rate and cholesterol degradation capacity.

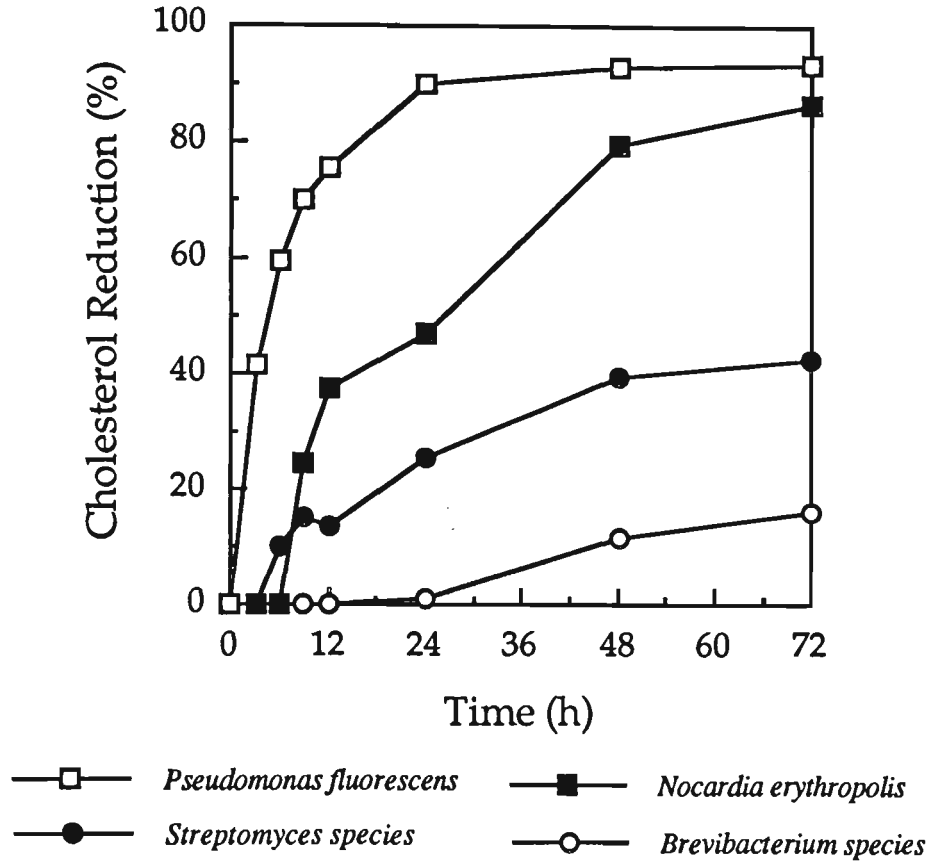


Figure 4.2 Time course of cholesterol degradation in egg yolk at 25°C by the four CODs investigated. Each data point is the average of triplicate samples.

The reaction of both Ne and Ss CODs occurred at a much lower rate for the first 48h at 25°C compared to that at 37°C. The cholesterol degradation capacity of both Ne and Ss CODs also decreased at 25°C with Ne COD degrading 86.4% and Ss COD degrading 42.3% of the total cholesterol after 72h. Bs COD showed little reaction until 24h incubation and 15.7% after 72h incubation. At 4°C, all enzymes, except Pf COD, showed no or insignificant reaction even after 72h of incubation (Figure 4.3). Interestingly, Pf COD could degrade approximately 30%

cholesterol within 3h and 67.2% after 48h, after which the cholesterol level remained almost unchanged.

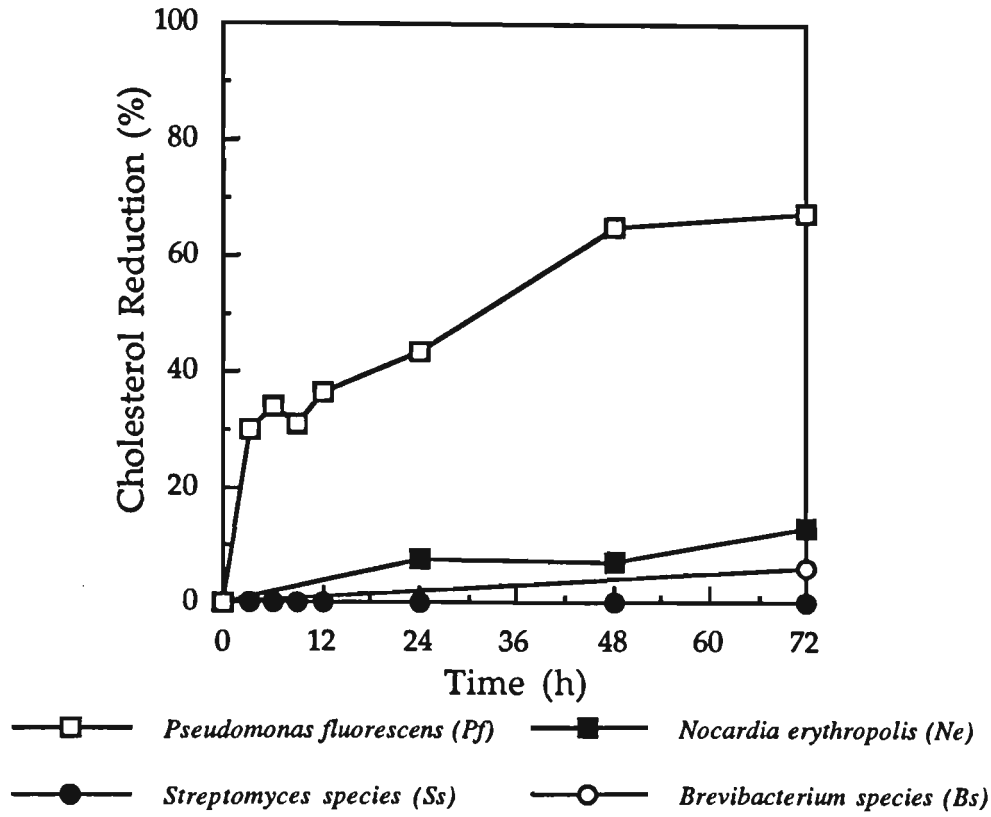


Figure 4.3 Time course of cholesterol degradation in egg yolk at 4°C by the four CODs investigated. Each data point is the average of triplicate samples.

In a previous study, Ss COD completely degraded free cholesterol in a buffer system within 24h at 25°C and 37°C but only 60% of cholesterol at 7°C or 45°C (Uwajima *et al.*, 1974). In milk, the Ss enzyme reduced about 35% of cholesterol after 10h at 25°C but up to 50% at 3°C and 7°C. By contrast, in the present egg yolk system, Ss COD showed high activity at 37°C (65%) and 25°C (35%) but no activity at 4°C. This observation may reflect the effect of substrate environment on the activity of the enzyme.

In a buffer system, Ss oxidases will readily reduce free cholesterol (100% reduction), but the membrane bound cholesterol present in milk is more difficult to reduce (35 to 50% reduction) (Jellema, 1980). In milk, cholesterol mainly exists in the globule surrounded by the milk fat membrane. To react with the cholesterol, the enzyme has to penetrate the membrane or the cholesterol has to be released. Ss cholesterol oxidase, may behave like some lipoproteins, with a tendency to attach to fat globules at low temperatures (Hung and Unger, 1993), thereby promoting the accessibility of cholesterol to the enzyme. This may explain why the reduction of cholesterol increased from 35% to 50% when the temperature was lowered from 35°C to 4°C. At the most active range of temperature (25°C to 37°C), Ss COD reacted with egg cholesterol as in a buffer system.

At 4°C, contrary to its behaviour with milk cholesterol, the Ss enzyme failed to react with egg cholesterol, possibly due to a lack of a common bonding site for both enzyme and cholesterol, which appears to be present in the milkfat membrane at low temperature (Uwajima *et al.* 1979). The results show that the cholesterol degrading capacity of these enzymes follows the sequence, Pf>Ne>Ss>Bs (Figures 4.1, 4.2 and 4.3). In a comparative study on CODs, Noma and Nakayama (1976) found that the degrading activity of these enzymes for pure cholesterol in a buffer system followed a reverse sequence Bs>Ss>Ne.

These observations appear to indicate that the activity of a COD is greatly influenced by the nature of the reaction system. For example, cholesterol oxidase isolated from Ss was found to be much more active in supercritical carbon dioxide, than in a buffer or yolk system. In supercritical carbon dioxide (100 bar, 35°C),

COD from Ss demonstrated 100% cholesterol degradation within 1h (Noma and Nakayama, 1976).

The ability of each of the four CODs to degrade egg yolk cholesterol, as a function of temperature, was measured by incubating each COD with egg yolk for 12h at temperatures ranging from 5°C to 60°C. The 12h incubation period chosen was based on data presented in the initial incubation studies (figures 4.1 to 4.3), which showed the majority of cholesterol being degraded in the first 12h. The results obtained from the temperature study are presented in figure 4.4.

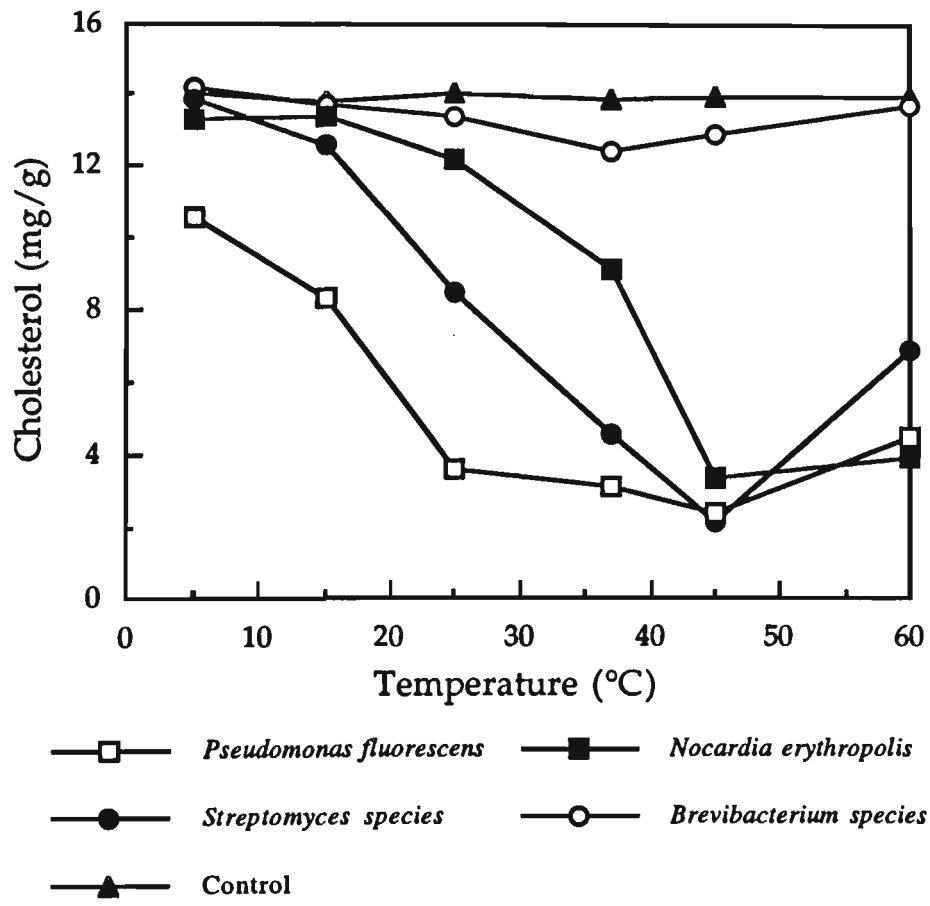


Figure 4.4 Effect of temperature on the activity of cholesterol oxidases from various CODs in egg yolk, after 12h. LSD (for comparing the control treatment at different temperatures)=0.58. LSD (for all other comparisons)=1.07. The experimental design is described in detail in Chapter 2.3.2.

The effect of temperature on the enzyme activity after 12h incubation differed greatly between enzyme types (interaction significant at $p<0.001$, Figure 4.4). An attempt was made to fit the treatment variation to linear, cubic and quadratic relationships, but none of these relationships could adequately account for all the variation among the treatment means (the lack of fit was significant, $p<0.001$).

The most effective temperature for the Pf, Ne and Ss CODs was around 45°C; for Bs COD maximum activity appeared closer to 37°C. The enzymes isolated from Pf, Ne and Ss showed good heat stability with substantial activity retained even at 60°C. The Pf COD has an effective temperature range from 5°C to 60°C, and from 5°C to 37°C it is clearly superior to the other three enzymes.

The most effective temperature/enzyme combination for maximum cholesterol degradation after 12h, was determined to be that by Pf COD incubated at 37°C (LSD=1.07 mg cholesterol/g). Raising the temperature to 45°C did not produce a significant change in the amount of cholesterol degraded by this enzyme, and heating further to 60°C was no different to incubating at 25°C for the same period (LSD=1.07 mg cholesterol/mg). At the low temperature of 5°C, Pf COD was the only enzyme to achieve a cholesterol reduction (up to 28.7%) which was significantly different to the control (LSD=0.88 mg cholesterol/g).

Effect of Concentration

The effect of enzyme concentration on cholesterol degradation is shown in figure 4.5. COD from Bs was not used due to the poor activity it demonstrated in the first two studies.

Based on data presented in figure 4.4, yolk samples were incubated at the optimum temperature of 45°C. An incubation time of 2h was chosen because the data presented in figure 4.1 indicates that the reaction order followed first order kinetics for this initial period.

The effect of enzyme concentration on the degradation of egg yolk cholesterol after 2h differed significantly (interaction significant as $p<0.01$, Figure 4.5).

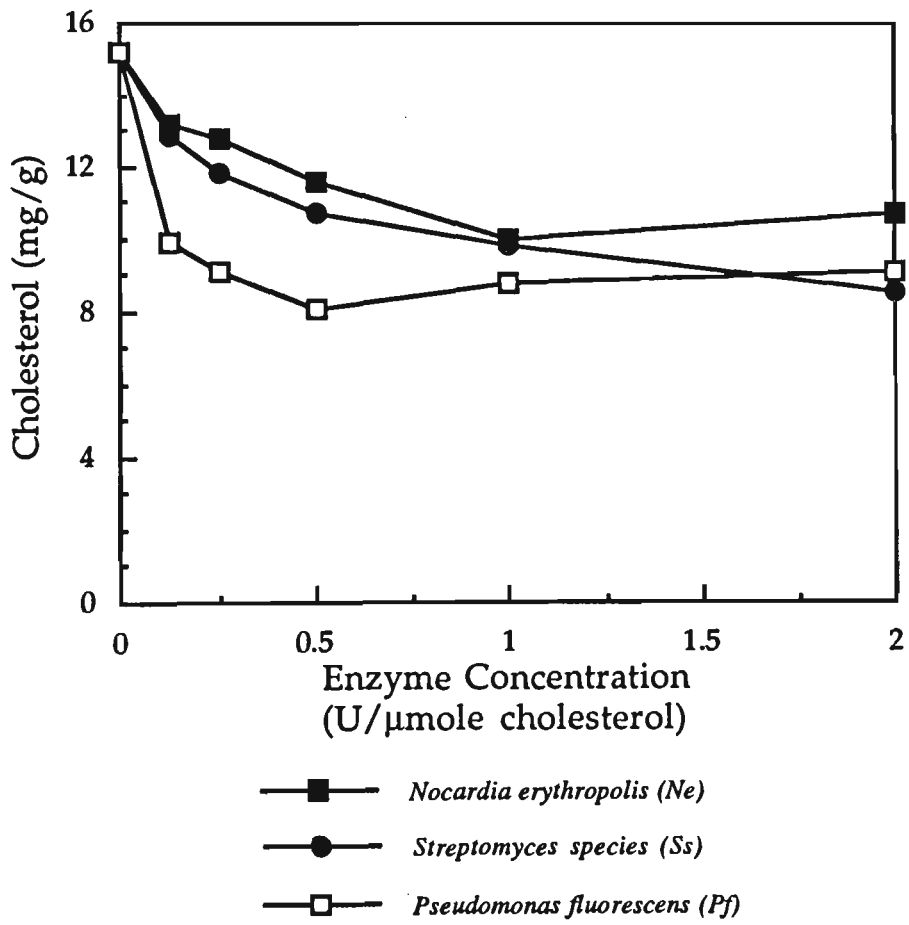


Figure 4.5 Effect of enzyme concentration on the degradation of cholesterol in egg yolk, at 45°C after 2h, by various CODs. LSD (for comparing control with non-control treatments at different concentrations)=0.41. LSD (for all other comparisons)=0.47. The experimental design is described in detail in Chapter 2.3.2.

As in the previous study on temperature effect, an attempt was made to fit the treatment variation to linear, cubic and quadratic relationships, but none of these relationships could adequately account for all the variation among the treatment means (the lack of fit was significant, $p<0.01$).

The most effective concentration of Pf COD and Ne COD was around 0.5 U/μM cholesterol and 1U/μM cholesterol, respectively. Increasing the concentration of either of these enzymes further did not produce significantly different reductions in cholesterol (LSD=0.47mg cholesterol/g). Surprisingly, even at the low concentration of 0.125 U/μmole cholesterol, COD from Pf degraded more than one third (up to 34.6%) of the egg cholesterol in 2h. This was the only significantly different reduction between treatments seen at this concentration (LSD=0.47mg cholesterol/g). Increasing the concentration of Pf COD further to 1 and 2 U/μM did not produce a reduction in cholesterol that was significantly different (LSD=0.47mg cholesterol/g).

Cholesterol degradation was significantly different to that of the control at all concentrations and enzyme types (LSD=0.41mg cholesterol/g). The most effective enzyme type/concentration combination was that of Pf COD at 0.5 U/μM.

Cholesterol Degradation Products

A number of COD products have been detected in various foods, including spray-dried egg yolk powder (Larsen and Froning, 1981). The adverse health effects of these oxidation products are still a topic for debate.

In this study, the degradation products of egg cholesterol were examined by TLC and confirmed by MSD. The only steroid-like component detected was 4-cholesten-3-one (R_f 0.65; MS m/e 384 (60 %, M), 207 (70), 124 (100)), for each of the four enzymes. The 4-cholesten-3-one has been described as the first degradation product of cholesterol oxidase catalysis in species of *Nocardia*, *Brevibacterium*, *Pseudomonas* and *Streptomyces* by many investigators (Aihara *et al.*, 1988a; Cheetham *et al.*, 1982; Flegg, 1973; Smith and Brooks, 1974; Tomioka *et al.*, 1976; Uwajima *et al.*, 1974).

In milk inoculated with Ss COD, 4-cholesten-3-one was formed but degraded further after 25h of incubation at both low (3°C to 7°C) and high temperature (25°C to 45°C) (Aihara *et al.*, 1988a). Aihara *et al.* (1988a) also found this compound in egg yolk inoculated with *R. equi* No. 23, a COD producing microorganism, but it was converted rapidly into non-steroid components.

CHAPTER 5

Microbial Degradation of Egg and Milk Cholesterol

5.1 Introduction

Cholesterol in milk and egg yolk can be degraded by isolated cholesterol oxidase enzymes (Xiansheng *et al.*, 1990), and by some intact microorganisms (Aihara *et al.*, 1988). Xiansheng *et al.* (1990) has demonstrated the ability of cholesterol oxidase isolated from *Streptomyces species* to completely degrade cholesterol in a buffer system at 25°C and 37°C, and up to 65% of the cholesterol in milk at lower temperatures (3-7°C), in less than 24h. Work described in Chapter 5 of this thesis indicates that up to 93% of egg yolk cholesterol is degraded by cholesterol oxidase from *Pseudomonas fluorescens* and *Streptomyces species* after incubation for 3d at 37°C and up to 64.9% with isolated COD from *Pseudomonas fluorescens* after incubation for 2d at 4°C.

The degradation of egg yolk cholesterol by *Rhodococcus equi* No. 23, a microorganism isolated from butter, has been described by Aihara *et al.* (1988a); an extracellular, membrane-bound form of the COD enzyme was identified in different strains of *Rhodococcus* with the highest levels of activity being produced by strain No. 23. This information was suggested as important for the industrial preparation of COD from culture filtrate, which can be used for the specific estimation of cholesterol in blood serum (Aihara *et al.*, 1986).

5.1.1 Equi Factor

The extracellular enzyme system secreted by *R. equi* No. 23 has been reported to contain an additional enzyme, phospholipase C, which encourages the degradation of cholesterol by cholesterol oxidase through decomposition of components in the

LDL-surface layer in egg yolk resulting in greater substrate availability. This cholesterol degrading system has been referred to as the "equi factor" (Aihara *et al.*, 1988b, Prescott *et al.*, 1982).

Moore *et al.* (1977) reported that digestion of LDL with phospholipase C increased the amount of lipid extracted by ether from 19% to 74%. The 10% of cholesterol that could be degraded without the digestion of phospholipid in the LDL-surface layer might indicate the presence of a small proportion of cholesterol which bound weakly with phospholipid, or existed in a more external position, than the major proportion of cholesterol.

Equi factor has been shown to be produced by 173 strains of serologically verified *Corynebacterium equi,* more commonly known as *R. equi* (Prescott *et al.*, 1982). However, the ability of *C. equi* to produce equi factor(s) should not be used as verification of the strain, since other organisms may produce similar effects, or there also may be a small number of *C. equi* strains which do not produce equi factor(s) (Prescott *et al.,* 1982).

Unfortunately, LDL plays an important role in the functional properties of egg yolk, such as emulsification and gelation, and therefore the lowering of these properties through LDL degradation by the enzymatic reaction is disadvantageous for the industrial uses of such treated egg yolk (Aihara *et al.*, 1988b). Further studies, for example using phospholipase C inhibitors, are needed to produce egg yolk LDL which contains less cholesterol than native LDL, but still allows the yolk to retain its functional properties (Aihara *et al.*, 1988b).

5.1.2 Cell Activity

On comparison with strains of the genera *Nocardia, Brevibacterium* and *Microbacterium* the rate of cholesterol degradation was much higher in strains of *Rhodococcus.* Some strains of *Rhodococcus erythropolis* can decompose between 90% and 100% of the cholesterol in various animal fats. The cholesterol content in chicken fat was reduced by 63% after 24h and by 97% after 72h of treatment with this organism (Watanabe *et al.*, 1986).

Johnson and Somkuti (1990) have reported that sonicated extracts of *R. equi* can degrade 40% of cholesterol in egg yolk and up to 2.4% in a milk cream preparation; the sonicated cell preparations were more effective in degrading free cholesterol than cholesterol in complex food systems such as milk cream and egg yolk.

The differences observed between *Rhodococcus* strains to degrade free cholesterol and cholesterol in egg yolk or milk cream could be due to (1) the presence of additional enzyme systems (esterase, phospholipase) contributing to the general destabilisation of cholesterol-binding structures in egg yolk and milk cream, thus resulting in greater substrate availability, and (2) the limited accessibility of cholesterol in egg yolk and milk cream to the enzymes of the *Rhodococcus* dissimilation system (Johnson and Somkuti, 1990).

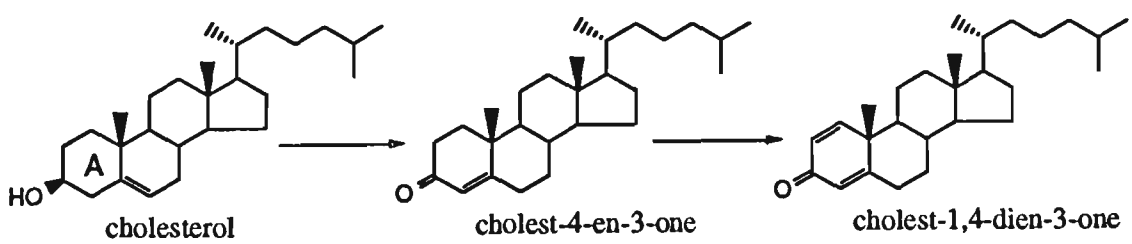
The activity of the cells has also been shown to be somewhat dependent on the culture growth medium. *R. equi* cells pre-incubated without cholesterol exhibited only 58% of the activity of cells pre-incubated with cholesterol, with pre-incubated

cells degrading nearly all of the cholesterol in a reaction system within 2h (Johnson and Somkuti, 1990).

5.1.3 Steroid Byproducts

The product of cholesterol dissimilation with *Rhodococcus* strains was found to be cholest-4-en-3-one, the first product of cholesterol degradation, confirming the presence of an oxidative pathway in this organism (Johnson and Somkuti, 1990). Cholestenone has also been reported as the sole steroidal product of cholesterol oxidation by *Rhodococcus* strains (Richmond, 1973; Aihara *et al.*, 1986). Sterol-decomposing microorganisms that accumulate only cholestenone as the degraded product, include bacteria, actinomycetes, moulds and yeasts (Watanabe *et al.*, 1989; Arima *et al.*, 1969)

Compared with the mammalian system where the A-ring is kept intact until cleavage of the side-chain, converting cholesterol into pregnenolone, is accomplished, microbial decomposition occurs at first in the A-ring, including dehydrogenation, isomerisation of a double bond, and Δ^1-dehydrogenation. Decomposition proceeds by the following pathway (Arima, 1969):



cholesterol cholest-4-en-3-one cholest-1,4-dien-3-one

5.1.4 Non -Steroid Byproducts

Some microorganisms which can degrade cholesterol without accumulating steroid intermediates have also been isolated (Aihara *et al.*, 1986, 1988; Noma and Nakayama, 1976, Johnson and Somkuti, 1990). When egg yolk was inoculated with *Rhodococcus equi*, up to 70% of cholesterol was degraded with almost no accumulation of steroid intermediates (Aihara *et al.*, 1988a).

Aihara *et al.* (1986) found that the amounts of cholest-4-en-3-one as an intermediate from cholesterol to lower degradation products differed among strains. Up to approximately 24h, cholesterol in the culture medium had been decomposed quantitatively to nearly zero at a high rate in the three strains, and 4-cholesten-3-one was formed as cholesterol decreased.

The virtual absence of other degradation products such as androst-1,4-dien-3,17-dione and androst-4-en-3,17-dione suggests that the cholest-4-en-3-one formed was quickly converted into non-steroid intermediates. This conversion rate with almost no accumulation of steroid intermediates in culture medium was previously demonstrated for the degradation of cholesterol by *Pseudomonas species* NCIB 10590 (Owen *et al.*, 1983).

The cholesterol degrading capacity of four strains of the genera *Rhodococcus* were investigated to determine their cholesterol degrading capacities in egg yolk and milk.

5.2 Results and Discussion

R. equi No. 23 is one of a number of organisms that utilise cholesterol as sole carbon and energy sources (Watanabe *et al.*, 1986). Some of these cholesterol degrading organisms are highly effective at utilising free cholesterol but much less so with cholesterol in complex food systems such as egg yolk and milk cream (Johnson and Somkuti, 1990).

Strains of *R. equi* No. 23 visible under oil immersion and high resolution (X 1000) all showed gram positive staining (purple colour). In all strains the morphogenetic cycle is initiated with the coccus or short rod stage (Goodfellow, 1984) (Figure 5.1).

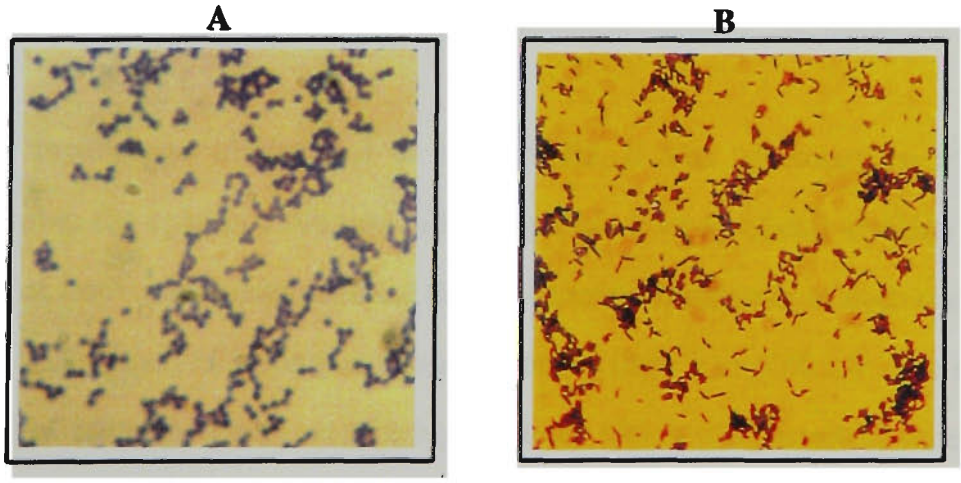


Figure 5.1 Photographs of (A) *R. equi* No. 23 cells as cocci and (B) *R. equi* No. 23 cells as rods under oil immersion (X 1000).

Cultures, *R. equi* No.23, *R. erythropolis* No. 3, *R. species* No.13 and *R. species* No.15 were grown in nutrient broth (Figure 5.2) and harvested at approximately 12, 45, 48 and 50h, respectively, for cholesterol degradation studies.

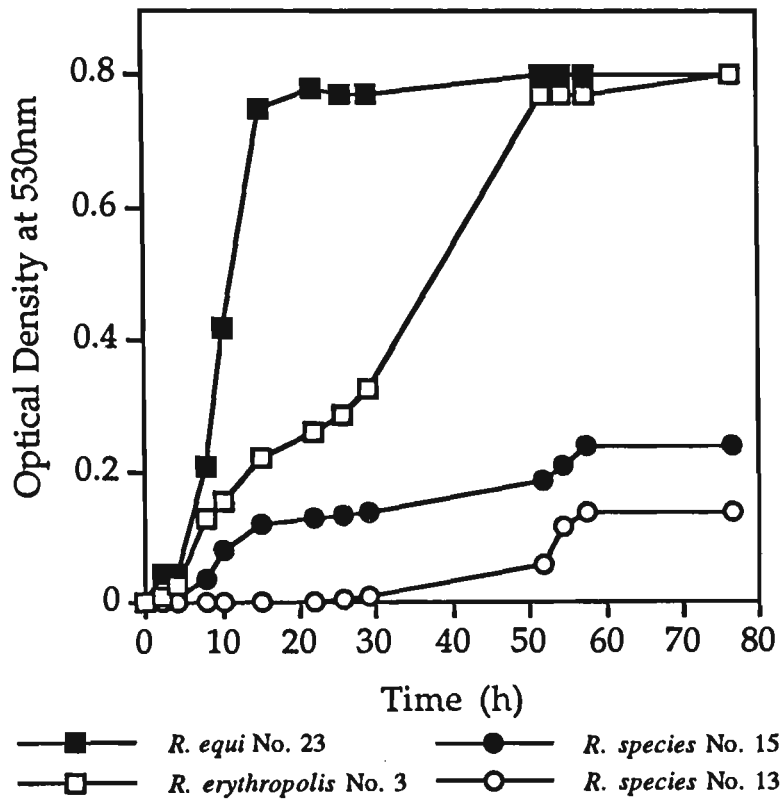


Figure 5.2 Turbidity measurements at 530nm of 10 mL cultures inoculated with 250µL stock culture over time with mechanical shaking, at 30°C

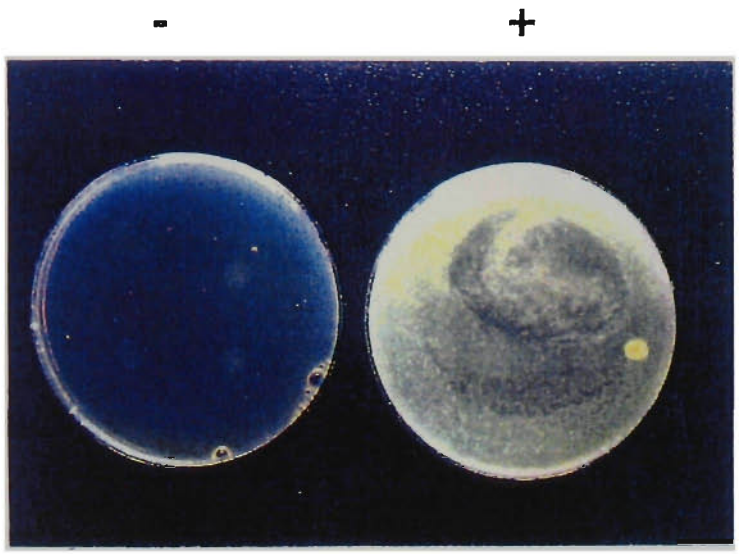
In order to disperse cholesterol in agar media, it was necessary to add the cholesterol to the agar as a solution in isopropanol. A study of cell viability in the presence of isopropanol demonstrated that the number of colonies of each organism declined as the level of isopropanol was increased from 0 to 50% v/v. The results showed that 5% isopropanol was the highest level which still permitted acceptable cell viability.

5.2.1 Detection of Cholesterol Degradation Activity in Agar Media

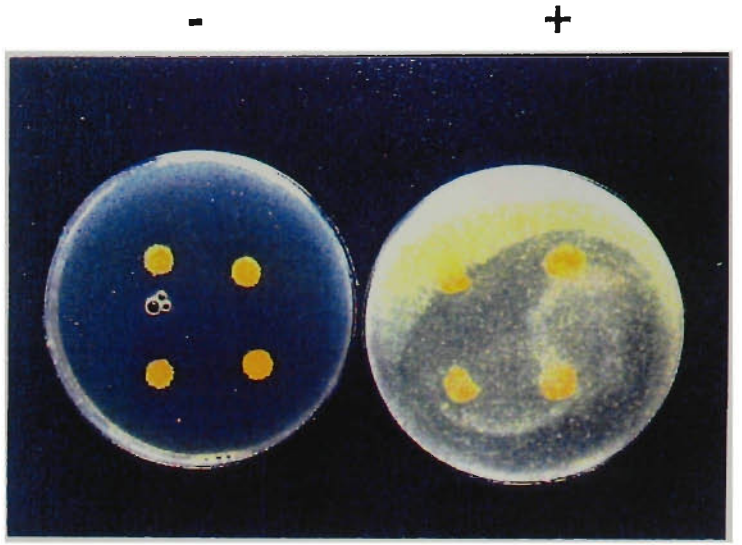
Halo formation around colonies grown on agar media containing added cholesterol was an indication of the conversion of cholesterol to cholest-4-en-3-one, the first product of cholesterol degradation, by the COD enzyme secreted by *Rhodococcus* organisms (Aihara *et al.*, 1986, Watanabe *et al.*, 1989).

Figure 5.3 shows the difference between the four strains in halo formation around colonies on the agar medium with and without added cholesterol. Activity was detected around colonies of *R. equi* No. 23 grown on agar medium with added cholesterol evident by the presence of a halo, but not around colonies grown on the cholesterol-free medium. A halo was not detected around colonies for the other three *Rhodococcus* strains on either media indicating little or no activity present. A higher production of cholesterol oxidase, both extracellular and membrane-bound, was found to be produced by *R. equi* No. 23 when compared to other strains of *Rhodococci* confirming an oxidative pathway in this organism (Aihara *et al.*, 1986, Watanabe *et al.*, 1989).

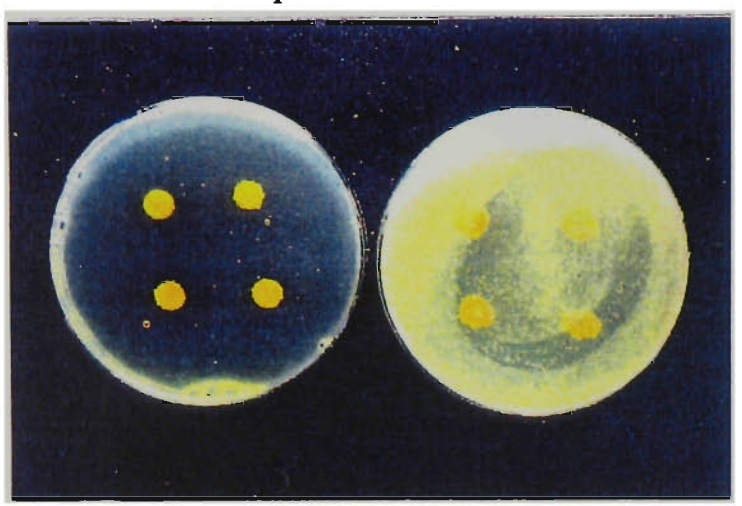
To examine the effectiveness of *R. equi* No. 23 in a complex food system, the organism was grown on agar containing egg yolk. A halo formation was observed around colonies of *R. equi* No. 23 grown on this medium (Figure 5.4). The percentage of remaining cholesterol in the vicinity of colonies of *R. equi* grown on yolk-containing media were determined. The remaining cholesterol in vicinity of the colony was 0.05mg/g (halo zone 1) and 1.02mg/g (halo zone 2) corresponding to a cholesterol reduction of 95% and 75%, respectively.



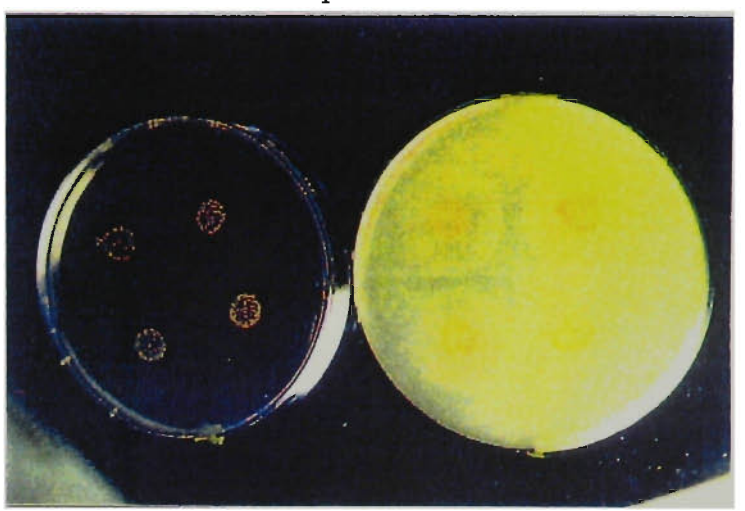
R. species No. 15



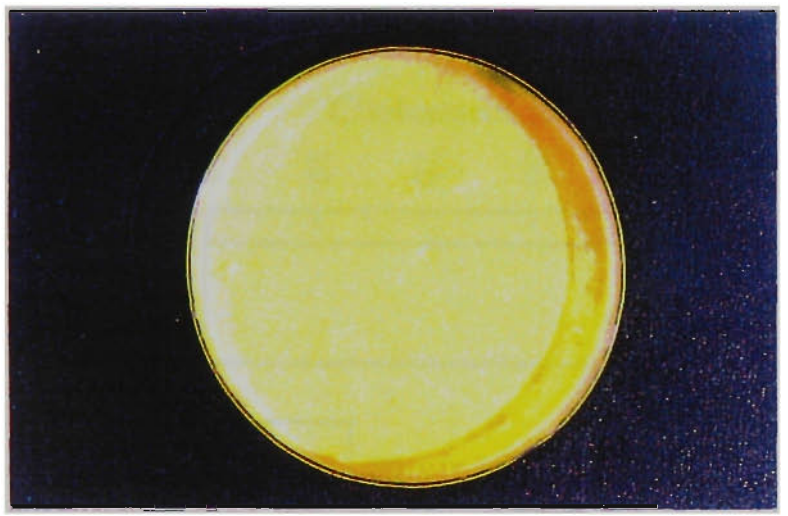
R. species No.13



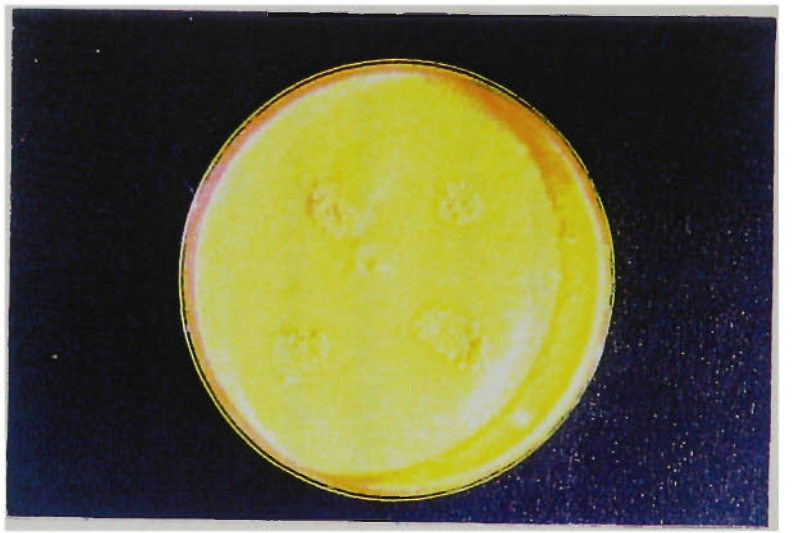
R. erythropolis No. 3



R. equi No. 23

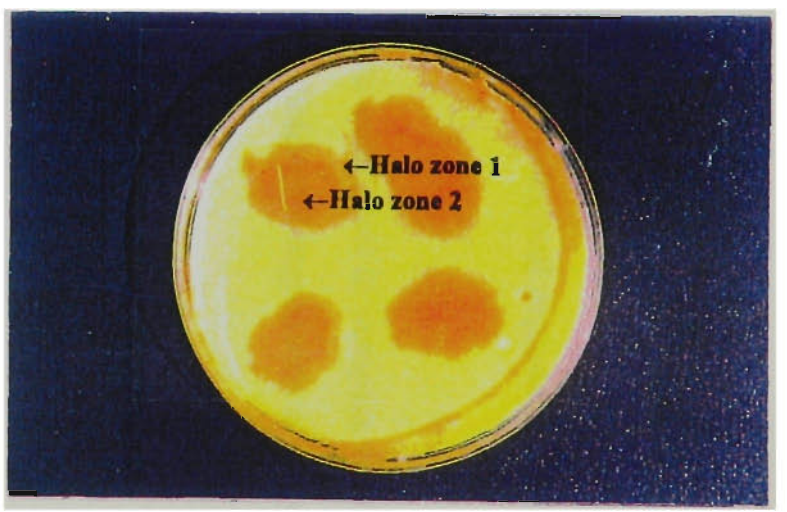


R. species No. 15



R. species No.13

R. erythropolis No. 3



R. equi No. 23

The degradation product, cholest-4-en-3-one, was detected by gas chromatography-mass spectrometry, confirming that cholesterol degradation had occurred within the halo zone.

Based on these observations further studies were conducted in complex food systems such as milk and egg yolk to examine the cholesterol degradation activity of *R. equi* No. 23.

5.2.2 Cholesterol Degradation in Milk

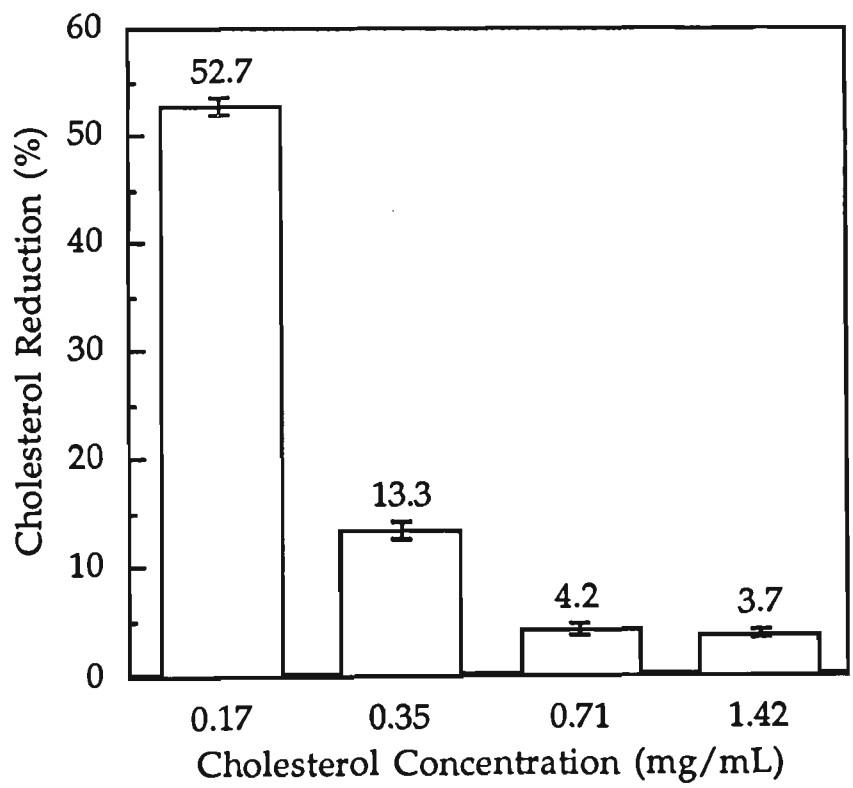


Figure 5.5 Cholesterol reduction in whole milk and in milk diluted, 1 in 2, 1 in 4, and 1 in 8, following incubation with *R. equi* No. 23 at 37°C for 7d.

Most strains of *Rhodococcus* have been reported to grow between 15°C and 40°C (Goodfellow, 1984). The cholesterol degrading capacity of *R. equi* No. 23 was determined in a milk system at 37°C for 7d (Figure 5.5). The results show that the percentage of cholesterol reduced decreased as the cholesterol substrate concentration increased from 0.17 to 1.42mg/mL.

Around 4% of cholesterol was degraded by *R. equi* No. 23 in whole milk (1.42mg/mL cholesterol) and in milk diluted 1 in 2 (0.71mg/mL cholesterol). Although the magnitude of degradation in both milks was small, the reduction in cholesterol was statistically significant in both the undiluted milk ($p<0.01$) and milk diluted 1 in 2 ($p<0.05$). When milk was diluted 1 in 4 (0.35mg/mL cholesterol) and 1 in 8 (0.17mg/mL cholesterol) cholesterol degradation increased to 13% ($p<0.05$) and 52% ($p<0.01$), respectively.

5.2.3 Effect of Culture Growth Medium on the Cholesterol Degrading Activity of Cells in Milk and Egg Yolk

The conditions under which cells are grown may affect their cholesterol degrading capacity. Aihara *et al.* (1988b) recommended that *R. equi* No. 23 should be pre-grown in cholesterol-containing medium to enhance the cholesterol reducing activity. Johnson and Somkuti (1990) also reported that cells grown in a cholesterol substrate have been induced to produce the cholesterol degrading enzymes required for cholesterol dissimilation.

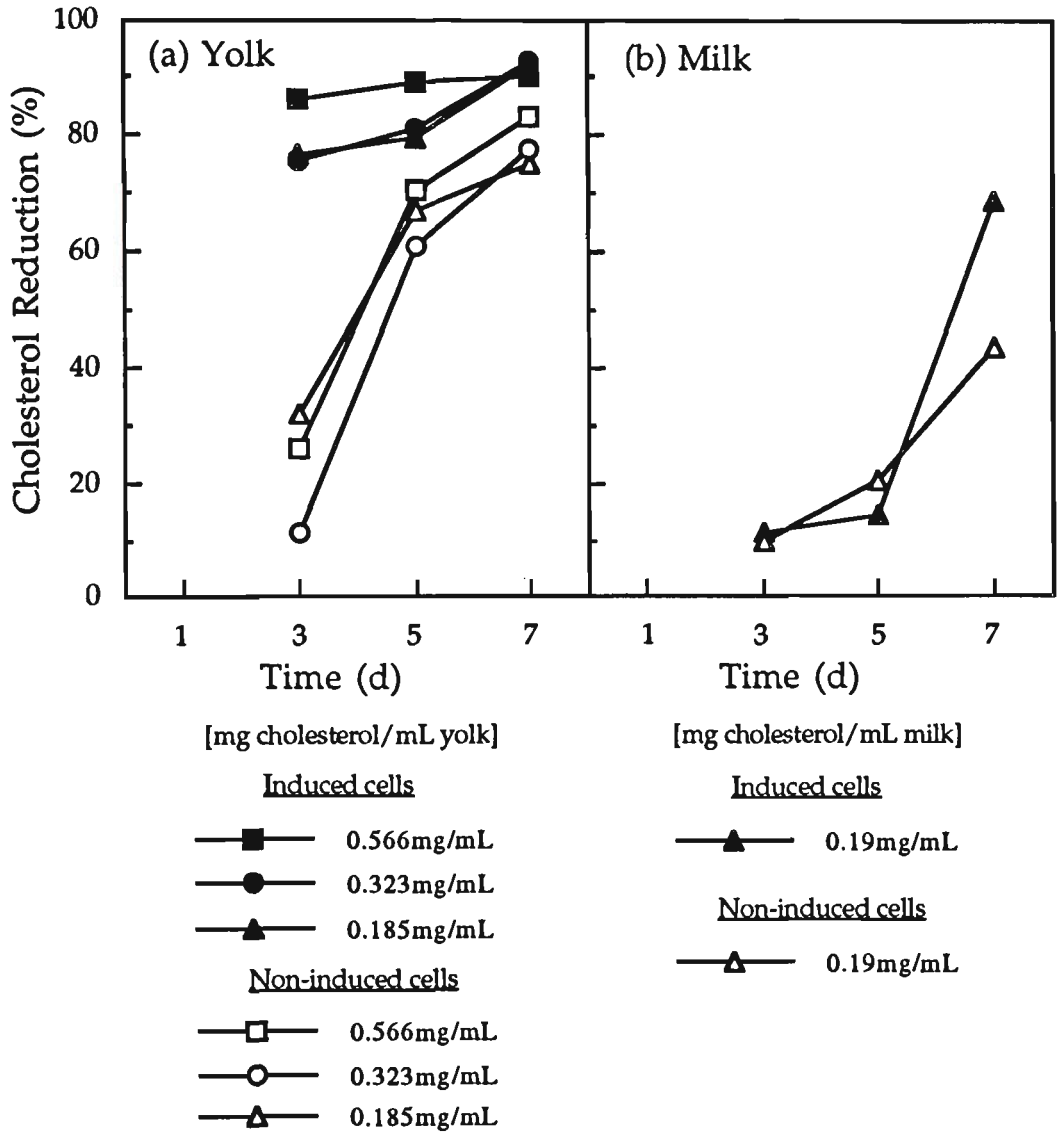


Figure 5.6 Time course of cholesterol degradation in (a) yolk and (b) milk, by induced and non-induced cells of *R. equi* No. 23

To examine the potential effect of growth conditions on the induction of cholesterol degradation activity, cells of *R. equi* No. 23 previously grown in the presence (induced) and absence of cholesterol (non-induced) were grown for 3, 5 and 7d at 37°C in milk and egg yolk. The cholesterol degrading capacity of these cells in a yolk system at 37°C for up to 7d is shown in figure 5.6(a). Induced cells of *R.*

equi No. 23 showed a higher level of cholesterol degrading activity in egg yolk than non-induced cells.

For each of the three yolk cholesterol concentrations investigated, more than 70% of cholesterol was degraded after 3d incubation with induced cells; the same level of degradation took 7d to achieve with non-induced cells. A similar result was obtained in a free cholesterol system (Johnson and Somkuti, 1990). The faster rate of cholesterol reduction by induced cells of *R. equi* No. 23 is likely to be due to a shortened lag phase in cell growth.

Cholesterol degradation was almost complete after 7d incubation with induced cells of *R. equi* No. 23 at the three cholesterol concentrations investigated, 0.57, 0.32 and 0.19mg/mL (90, 92 and 92%, respectively) (Figure 5.6(a)); there was no significant difference ($p>0.05$) between the amounts of cholesterol degraded after 7d incubation for each of the cholesterol concentrations. Similarly, the amounts of cholesterol degraded by non-induced cells of *R. equi* No. 23 at 7d, for each cholesterol level, did not differ significantly ($p>0.05$).

The cholesterol degrading capacity of non-induced and induced cells of *R. equi* No. 23 in a milk system at 37°C after 7d is shown in figure. 5.6(b). A greater reduction in cholesterol was observed in milk inoculated with induced cells of *R. equi* compared to milk inoculated with non-induced cells, 68% and 43%, respectively.

5.2.4 A Comparison of Milk and Egg Yolk Cholesterol Degradation

The cholesterol degrading system of *R. equi* No. 23 was effective at degrading both milk and yolk cholesterol. In milk, cholesterol degradation was greater at the lower levels of cholesterol investigated. However, it was only at very low milk cholesterol concentrations (0.17mg/mL) that a substantial amount of cholesterol was degraded (>50% after 7d). This was in sharp contrast to egg yolk where a significant amount of cholesterol was degraded at levels as high as 0.57mg/mL (>80% after 7d).

One interpretation of these results is that the COD enzyme had become saturated at a lower substrate concentration in milk than in egg yolk. All enzymes show the saturation effect, but vary widely with respect to the substrate concentration required to produce the effect (Lehninger, 1975a). Alternatively, the cholesterol in milk may have been less accessible to the cholesterol dissimilating system of *Rhodococci* than cholesterol in egg yolk. A similar hypothesis was proposed by Johnson and Somkuti (1990) in their investigations with yolk and milk cream.

5.2.5 Products of Cholesterol Degradation in Egg Yolk

The changes in the degradation products of yolk cholesterol were followed by TLC. Though diminishing amounts of cholesterol (R_f 0.47) were observed as the cholesterol level was lowered, degradation products were not detected in the media containing 0.19mg/mL and 0.32mg/mL cholesterol. These results may suggest that if any steroids were formed, they were rapidly converted to non-steroid products

without accumulation of steroid intermediates. However, minute amounts of the degradation product cholest-4-en-3-one were detected by GC-MS in media containing 0.56mg/mL yolk cholesterol only. Cholest-4-en-3-one was characterised by the molecular ion at m/e 384 (46%, M) and fragment ions at 229 (57) and 124 (100). The absence of steroid intermediates was also reported by Aihara *et al.* (1986, 1988) in their investigation of cholesterol degradation by *R. equi* No. 23 in egg yolk and a pure cholesterol system.

CHAPTER 6

Conclusions

6.1 Development of a Rapid Cholesterol Assay

Foods in which cholesterol occurs include food products with different and complex matrixes. This presents problems in determining accurate values for cholesterol. A modified method of Kovacs (1990) using solvent extraction and gas chromatographic determination was developed and used to measure cholesterol in eggs. The results obtained in this study indicated that there was less variation and better repeatability among replicate extractions with the modified technique. The modified procedure was superior to the original method since both higher cholesterol values and higher cholesterol recoveries were obtained, suggesting more effective cholesterol extraction. The modified procedure requires less solvent and less time than the original procedure.

Increased public awareness to the effect of cholesterol on CHD and the relationship of blood cholesterol to the diet has made it necessary to have a rapid, reliable and accurate method to satisfy nutritional labelling demands (Al-Hasani *et al.*, 1990). The method employed appears to provide a greater specificity and accuracy in analysing food with different and complex matrixes.

6.2 Cholesterol Content in Foods

The effectiveness with which cholesterol was extracted from egg yolk by the modified extraction method was also effective in extracting cholesterol from more complex food matrixes. The modified extraction procedure was used to measure the cholesterol levels in a number of foods. The accuracy of the modified procedure to measure cholesterol in other food matrixes, however, was not

demonstrated as reproducibility and recovery studies were not undertaken. The modified procedure successfully demonstrated the ability to extract cholesterol from non-egg based foods as well as egg-based foods.

6.3 Cholesterol Degrading Ability of COD in Egg Yolk

The cholesterol degrading capacity of four COD enzymes has been investigated in egg yolk, a complex food system. All enzymes catalysed the transformation of cholesterol to cholest-4-en-3-one. More than 50% of egg cholesterol could be reduced by three CODs (Ne, Pf and Ss), within 24 hours. The Pf COD was effective over a wider temperature range (4-60°C) than the other enzymes studied. The low concentration of COD from Pf required to achieve significant reduction of yolk cholesterol, coupled with the ability of this enzyme to degrade cholesterol at 4°C, suggests that this enzyme may provide an economical and practical approach for lowering the cholesterol level in egg yolk on a commercial scale.

6.4 Cholesterol Degrading Ability of Four Microorganisms in Egg Yolk and Milk

The cholesterol degrading ability of four microorganisms was investigated in both whole milk and egg yolk. Of the organisms studied *R. equi* was the only one effective in degrading cholesterol; this was evident by halo formation about colonies of *R. equi* grown on cholesterol-containing agar medium. The investigations revealed that *R. equi* was effective in reducing cholesterol in yolk but much less so in milk. In egg yolk, a substantial proportion of cholesterol was degraded (>70%) after 7d at each of the three cholesterol levels studied. After the

same period of incubation, milk cholesterol at a level comparable to the lowest concentration of yolk cholesterol (0.19mg/mL), was only degraded by 44%. In milk, appreciable cholesterol reduction was only observed at the lowest level of cholesterol studied; at the higher levels (0.35-1.42mg/mL) cholesterol reduction was less than 15%.

The culture growth medium of the *R. equi* inoculum had a marked affect on the magnitude of cholesterol degradation in both food matrixes. Cells harvested from cultures pre-grown in the presence of cholesterol, degraded more cholesterol in both milk and egg yolk than cells pre-grown in the absence of cholesterol. After 3d incubation, induced cells in egg yolk degraded up to 7 times the amount of cholesterol at the lower cholesterol level (0.19mg/mL), and up to 4 times the amount at the highest cholesterol level (0.57mg/mL), than non-induced cells. Similarly, cholesterol reduction in milk was enhanced when milk was inoculated with induced cells of *R. equi*, although the increased cholesterol degradation by induced cells was only evident after 7d incubation.

The results of the present work suggest that, like the COD from *Pseudomonas fluorescens*, the COD from *Rhodococcus equi* No.23 may have application to the commercial preparation of reduced cholesterol egg-based products for the food industry. The COD from *P. fluorescens* was found to degrade cholesterol at temperatures as low as 4°C; the ability of the COD from *R. equi* to degrade cholesterol at low temperatures remains to be investigated.

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Journal of Food Protection, Vol. 57, No. 10, Pages 908-912

Enzymatic Degradation of Egg Yolk Cholesterol

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(Received November 22, 1993/Accepted May 13, 1994)

ABSTRACT

The capacity of four cholesterol oxidases from *Nocardia erythropolis* (Ne), *Streptomyces* species (Ss), *Brevibacterium* species (Bs) and *Pseudomonas fluorescens* (Pf) to degrade cholesterol in egg yolk at 4, 25 and 37°C has been determined. The effects of enzyme/substrate concentration and incubation temperature on the enzymatic activity of these cholesterol oxidases have also been investigated. The cholesterol degrading capacity of these enzymes follows the sequence Pf>Ne>Ss>Bs. Up to 93.4% cholesterol was degraded by cholesterol oxidases from *P. fluorescens* and *Streptomyces* species after 72 h of incubation at 37°C. At 4°C, *P. fluorescens* cholesterol oxidase could degrade up to 64.9% cholesterol after 48 h.

Key Words: Enzymatic degradation, eggs, cholesterol.

High cholesterol content in egg is a factor contributing to declining egg consumption (*27*). A suspected causable association between dietary cholesterol intake and the development of coronary heart disease is still a topic for debate (*30*). Several studies have also indicated no or insignificant association of cholesterol intake from egg with serum cholesterol (*11,17,32*). Nevertheless, to address this cholesterol concern, the egg industry has attempted to produce low cholesterol eggs as well as egg products by various approaches.

Genetic selection to develop a chicken line which could produce low cholesterol eggs has only yielded moderate results (2.4% cholesterol reduction) (*13,22*). Modification of the hen's feed by dietary lipid treatment or by adding garlic oil did not influence cholesterol concentration in the egg (*26,36*). Consequently, cholesterol reduced egg products are prepared by rather complex processes (for example, by partially replacing egg yolk with vegetable oil or water then combining with egg white to meet the market demand) (*4,8*). The recombined egg does not retain the desirable egg flavor. Therefore, there is a need for a simple technique to produce cholesterol-free or cholesterol reduced egg yolk without losing egg flavor, and as a food ingredient for various egg based products.

Extraction of egg cholesterol using organic solvents has met with limited success due to a large amount of cholesterol remaining in the extracted egg as well as some undesirable effects on the functional properties of the egg yolk (*20*). Recently, β-cyclodextrin has been used to remove egg cholesterol by the formation of an insoluble complex with cholesterol or its ester in an aqueous solution. However, even with an additional enzymatic treatment it is still difficult to completely remove all residues of β-cyclodextrin from the yolk (*9*). This is a major impediment since β-cyclodextrin is not an approved food ingredient in some countries.

Supercritical fluid extraction could provide another means to remove egg cholesterol. In spite of the high capital investment required, this process already has several commercial applications in the food industry. Although up to 80% of egg cholesterol can be removed by supercritical fluid extraction using carbon-dioxide as the fluid, an economical technique to recover the cholesterol from the significant amount of co-extracted egg lipid is required as a prerequisite for commercial feasibility (*12,14*).

Microbiological degradation of cholesterol by some strains of *Nocardia, Rhodococcus, Brevibacterium, Corynebacterium* or *Streptomyces* has been reported (*1,2*). However, some of these organisms may accumulate steroid intermediates with consequent adverse health effects (*3*). Some strains which could degrade cholesterol without accumulating steroid intermediates have also been isolated (*3*). When egg yolk was inoculated with *Rhodococcus equi*, up to 70% of cholesterol was degraded with almost no accumulation of steroid intermediates (*2*).

Johnson and Somkuti also found *Rhodococcus* strains could reduce up to 80% of egg cholesterol (*16*). Although these findings indicate that enzymatic treatment could be used for egg cholesterol degradation, for any commercial application it would be necessary to shorten the degradation time of 3 days reported in these studies.

In a previous study, we reported that cholesterol oxidase could degrade all cholesterol in a buffer system and up to 65% in milk at low temperature (3 to 7°C), in less than 24 h (*36*).

This paper reports cholesterol degradation activities of cholesterol oxidases originating from four different microorganism genera, *Brevibacterium, Nocardia, Pseudomonas* and *Streptomyces*, in an egg yolk system under different environments in order to define the optimal conditions for each enzyme.

MATERIALS AND METHODS

Egg yolk.

Yolks and whites from 61 eggs (weighing 55 g each) were carefully separated. The yolks were combined and mixed until homogenous. A 0.2 g/ml yolk suspension in 0.1 M potassium phosphate buffer (KPB) pH 7 containing 0.02% thimerosal, as an anti-bacterial agent, was portioned into 20 ml lots and stored at -4°C until required.

Cholesterol oxidases.

Cholesterol oxidases (COD) derived from Ss, Bs, Ne and Pf were purchased from Sigma Chemical Company, St. Louis, MO as lyophilized powders. Cholesterol oxidases from Ne was reconstituted in 0.1 M KPB, pH 5 and the others in the same buffer but at pH 7. These pH's have been reported as being the most stable environment to store these enzymes to prevent appreciable loss of activity (*28*).

Since the incubation pH may have a strong effect on the enzymatic activity, the degradation capacity of each enzyme was measured at pH 7, a pH at or close to their optimum physiological pH based on available literature. Cheillan et al. (*6*) observed activity by Pf COD remaining constant as long as the pH was maintained between 6.5 and 8.5. Uwajima et al. (*33*) have shown that the enzyme isolated from *Brevibacterium* had a broad pH optimum in the range of pH 6.0 to 8.5 and Noma and Nakayama (*23*) observed pH optimum of 7.0 for Ne and Bs COD, and pH 7.5 for enzyme from *Streptomyces*.

For enzyme activity, one enzyme unit (1 U) is defined as the amount of COD that will promote the conversion of 1 µM of cholesterol to cholest-4-en-3-1 per min under the prescribed test conditions (*6*).

Cholesterol determination.

Cholesterol was determined by gas chromatography, following a method of Kovacs (*18*) with some modifications (*7*), with the principal change being that samples containing 1 to 2 mg cholesterol were converted to trimethylsilyl (TMS) derivatives prior to gas chromatography, by heating with 100 µl MSTFA (Sigma) at 100°C for 15 min. Octacosan-1-ol or dihydrocholesterol were used as internal standards. Coefficient of variation for the reproducibility and the mean recovery of the method used ranged from 2.2 to 2.9% and 90.2 to 95%, respectively.

A 25QC2/BP10-0.25 capillary column was used in a Hewlett Packard 5890 gas chromatograph. Column temperature was set at 285°C and injection temperature at 300°C. A flame ionisation detector was used, and this was set at 320°C. A split injection (1 µl) was made with a split ratio of 30:1. Flow rate of the carrier gas (He) was 0.8 ml/min and the head pressure set at 24 psi. The run time was 17 min, with the retention times of the TMS derivatives of cholesterol and dihydrocholesterol being typically 10 and 10.3 min, respectively.

Sterols were identified by co-chromatography using TMS derivatives of commercially purchased standards (Sigma).

Cholesterol degradation products.

In order to detect COD products, thin-layer chromatography (TLC) was performed with pre-coated silica Gel 60 F_{254} plates (Merck). Incubation products were separated with a mixed solvent system (chloroform: diethyl ether [60:40]) at room temperature and spots were made visible by spraying with 2',7'-dichlorofluorescein and viewed under ultra-violet (UV) light (254 nm). Standards of cholesterol and possible oxidation by-products (5-cholesten-3-one, 4-cholesten-3-one, 25-hydroxycholesterol and 7-ketocholesterol) were purchased from Sigma.

The identification of by-products was confirmed using gas chromatographic retention times in conjunction with mass spectra obtained. Incubation products were extracted with diethyl ether (2 ml + 1 ml), the extracts evaporated to dryness and the residues dissolved in chloroform (500 µl). Samples (1 µl) were then taken for GC-MS using a Hewlett Packard 5890 gas chromatograph fitted with a Hewlett Packard 5971A Mass Selective Detector (MSD). The column and chromatographic conditions were the same as for cholesterol determination.

Enzymatic treatment of egg yolk cholesterol.

Effect of time and temperature. The effect of incubation time on the cholesterol degrading capacity of each enzyme was initially investigated. Yolk suspensions containing 0.1 g yolk (approximately 3.9 µM cholesterol) were added to tightly capped (teflon-lined) 10 ml test tubes and incubated, in triplicate, with 3.9 U of each enzyme at 4, 25 and 37°C. Residual cholesterol was determined at 3, 6, 9, 12, 24, 48 and 72 h by gas chromatography. The experiment was not replicated. Results of this investigation were used to select 12 h as a suitable time for a more detailed study of incubation temperature.

To four of seven test-tubes containing 0.1 g yolk in buffer suspension, 3.9 U of COD enzyme from either one of four types (Ne, Ss, Bs and Pf) was added. The other three test-tubes contained no enzyme and were treated as controls. These treatments were randomly positioned into test-tube racks, and these racks were randomly assigned to incubators at each of the temperatures, 5, 15, 25, 37, 45 and 60°C. Incubation time was 12 h.

The experiment was replicated on three different days and described as a split-plot type design (*29*). Days were considered as blocks, incubators as plots and test-tubes containing yolks as the sub-plots. Temperature was the main plot treatment and the enzymes (including controls) were the sub-plot treatments. The main temperature effect, and the temperature by enzyme type interaction, were divided into orthogonal polynomial components (*29*) up to cubic order to determine if there were linear and quadratic trends. The data was analyzed as a temperature by enzyme type factorial with the control being considered in the analysis as an enzyme type. Pairs of means were compared using Fisher's least significant difference (LSD) at the 5% level.

Effect of concentration. Yolk suspensions containing 0.1 g yolk cholesterol were incubated with 0, 0.5, 1, 2, 4 and 8 U of enzyme per µM egg cholesterol for 2 h at 45°C and residual cholesterol was determined. The analysis was replicated on 3 days, with each day considered as a block (*29*). Within each block there was one test-tube sample of each enzyme type by concentration combination, plus two control samples (without enzyme). The arrangement of the test-tubes placed in the incubator was fully randomized. The data was analyzed as an enzyme type by concentration factorial plus an external non-enzyme control treatment, with blocking for time. Within the factorial, the concentration effects and concentration by enzyme type interaction were divided into orthogonal polynomial components (*29*) up to cubic order to determine if there were linear and quadratic trends. Pairs of means were compared using LSD at the 5% level.

RESULTS AND DISCUSSION

Cholesterol content of treated and untreated egg yolks were compared and results expressed as either percent of cholesterol reduction or mg/g residual cholesterol.

Effect of time and temperature.

The time course of cholesterol degradation of four COD in egg yolk at 37, 25 and 4°C are shown in Fig. 1, 2 and 3 when the yolk was incubated with the enzymes for 72 h.

The cholesterol degradation activity of each enzyme at 37°C is presented in Fig. 1. At 37°C, the Bs COD could

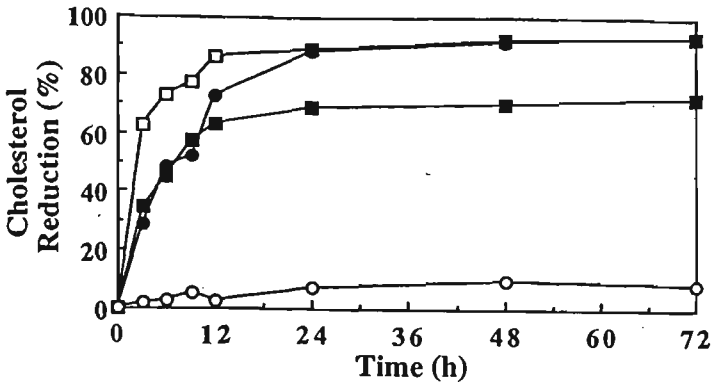

Figure 1. *Time course of cholesterol degradation in egg yolk at 37°C by COD of Pf (□), Ne (■), Ss (●) and Bs (○).*

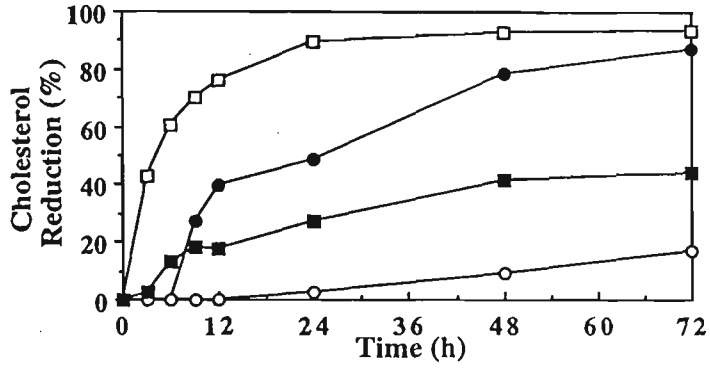

Figure 2. *Time course of cholesterol degradation in egg yolk at 25°C by COD of Pf (□), Ne (■), Ss (●) and Bs (○).*

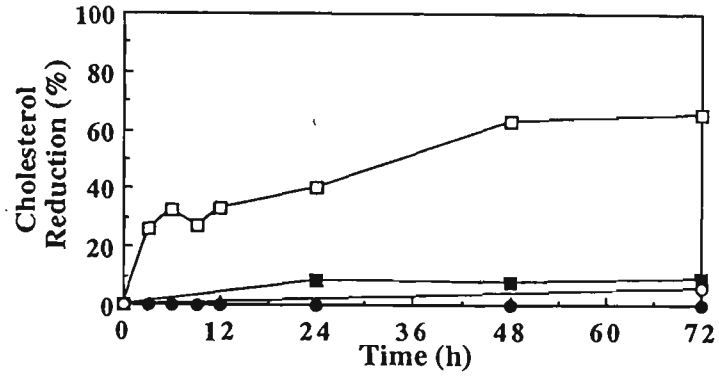

Figure 3. *Time course of cholesterol degradation in egg yolk at 4°C by COD of Pf (□), Ne (■), Ss (●) and Bs (○).*

only degrade about 8.3% cholesterol after 72 h of incubation while Ss COD reduced cholesterol 72.6%. Both Pf COD and Ne COD, respectively, reduced up to 93.4% of the cholesterol.

With the exception of Bs COD, cholesterol degradation at 37°C occurred in two stages. In the first stage, the reaction proceeded rapidly up to approximately 12 h. In the second stage, the reaction rate became slower, and after 24 h remained almost unchanged.

The performance of the Pf COD at 25°C (Fig. 2) was similar to that at 37°C in terms of reaction rate and cholesterol degradation capacity. The reaction of both Ne and Ss CODs occurred at a much lower rate for the first 48 h at 25°C as compared to that at 37°C. The cholesterol degradation capacity of both Ne and Ss CODs also decreased at 25°C with Ne COD degrading 86.4% and Ss COD 42.3% cholesterol after 72 h. *Brevibacterium* species cholesterol oxidase showed no reaction after 48 h incubation and 15.7% after 72 h incubation. At 4°C, all enzymes, except Pf COD, showed no or insignificant reaction even after 72 h of incubation (Fig. 3). Interestingly, Pf COD could degrade approximately 30% cholesterol within 3 h and 67.2% after 48 h, after which the cholesterol level remained almost unchanged.

In a previous study, Ss COD completely degraded cholesterol in a buffer system within 24 h at 25 and 37°C but only 60% of cholesterol at 7 or 45°C (*36*). In milk, the Ss enzyme reduced about 35% of cholesterol after 10 h at 25°C but up to 50% at 3 and 7°C. By contrast, in the present egg yolk system Ss COD showed high activity at 37°C (65%) and 25°C (35%) but no activity at 4°C. This observation may reflect the effect of substrate environment on the activity of an enzyme.

In a buffer system, Ss oxidases will readily reduce free cholesterol (100% reduction), but the membrane bound cholesterol present in milk is more difficult to reduce (35 to 50% reduction) (*15*). At the most active range of temperature (25 to 37°C), Ss COD reacted with egg cholesterol as in a buffer system. At 4°C, contrary to its behavior with milk cholesterol, the Ss enzyme failed to react with egg cholesterol, possibly due to a lack of a common bonding site for both enzyme and cholesterol, which appears to be present in the milkfat membrane at low temperatures (*36*). The results show that the capacity for yolk COD by these enzymes followed the sequence Pf>Ne>Ss>Bs (Fig. 1, 2 and 3). In a comparative study on CODs, Noma and Nakayama (1976) (*23*) found the degrading activity of these enzymes for pure cholesterol in a buffer system followed a reverse sequence Bs>Ss>Ne. These observations appear to indicate that the activity of a COD is greatly influenced by the nature of the reaction system. For example, cholesterol oxidase isolated from Ss was found to be much more active in a reaction system of supercritical fluid carbon-dioxide, than in a buffer or yolk system. In supercritical carbon dioxide (100 bar, 35°C), COD from Ss demonstrated 100% cholesterol degradation within 1 h (*25*).

The 12-h incubation chosen to investigate the effect of temperature on cholesterol degrading ability by all four enzymes was based on data presented in initial incubation studies (Fig. 1- 3), which showed the majority of cholesterol being degraded in the first 12 h (Fig. 4). The effect of temperature on the enzyme activity after 12-h incubation differed greatly between enzyme types (interaction significant at $p<0.001$, Fig. 4). An attempt was made to fit the treatment variation to linear, cubic and quadratic relationships, but none of these relationships could adequately account for all the variation among the treatment means (the lack of fit was significant, $p<0.001$).

The most effective temperature for Bs COD and that of the other CODs was around 37 and 45°C, respectively. The enzymes isolated from Pf, Ne and Ss showed good heat stability with substantial activity retained even at 60°C. The Pf COD has an effective temperature range from 5 to 60°C, and from 5 to 37°C it is clearly superior to the other three enzymes.

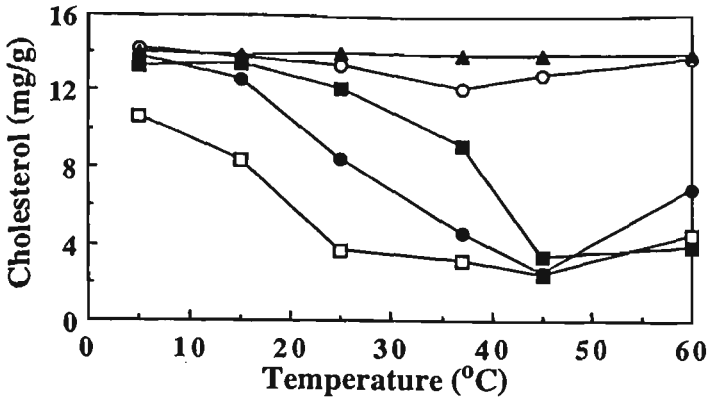

Figure 4. *Effect of temperature on the activity of cholesterol oxidases from Pf (□), Ne (■), Ss (●) and Bs (○) in egg yolk, after 12 h. Mg/g residual cholesterol for control (▲). LSD (for comparing the control treatments at different temperatures) = 0.58. LSD (for all other comparisons) = 1.07.*

The most effective temperature/enzyme combination for maximum cholesterol degradation after 12 h, was determined to be that by Pf COD incubated at 37°C (LSD = 1.07 mg cholesterol/g). Raising the temperature to 45°C did not produce a significant change in the amount of cholesterol degraded by this enzyme, and heating further to 60°C was no different to incubating at 25°C for the same period (LSD = 1.07 mg cholesterol/g). At the low temperature of 5°C, Pf COD was the only enzyme to achieve a cholesterol reduction (up to 28.7%) which was significantly different to the control (LSD = 0.88 mg cholesterol/g).

Effect of concentration.

The effect of enzyme concentration on cholesterol degradation is shown in Fig. 5. COD from Bs was not used due to the poor activity it demonstrated in the first two studies.

Based on data presented in Fig. 4, yolk samples were incubated at the optimum temperature of 45°C. An incubation time of two hours was chosen because the data presented in Fig. 1 indicates that the reaction order followed first order kinetics for this initial period.

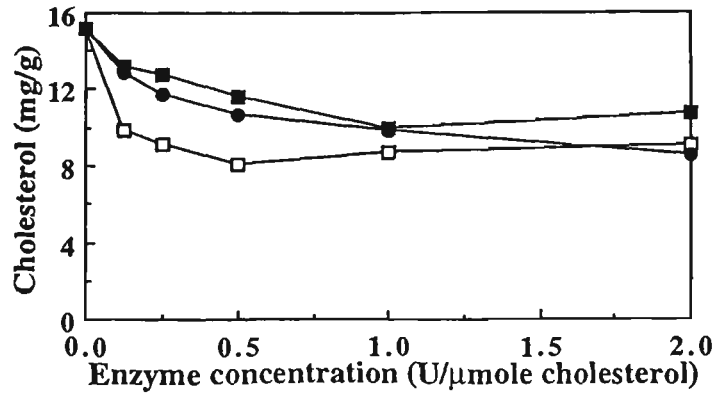

Figure 5. *Effect of enzyme concentration on the degradation of cholesterol in egg yolk, at 45°C after 2 h. Cholesterol oxidase from Pf (□), Ne (■) and Ss (●). LSD (for comparing control with non-control treatments at different concentrations) = 0.41. LSD (for all other comparisons) = 0.47.*

The effect of enzyme concentration on the degradation of egg yolk cholesterol after 2 h differed significantly (interaction significant as $p<0.01$, Fig. 5). As in the previous study on temperature effect, an attempt was made to fit the treatment variation to linear, cubic and quadratic relationships, but none of these relationships could adequately account for all the variation among the treatment means (the lack of fit was significant, $p<0.01$).

The most effective concentration of Pf COD and Ne COD was around 0.5 U/μM cholesterol and 1 U/μM cholesterol, respectively. Increasing the concentration of either of these enzymes further did not produce significantly different reductions in cholesterol (LSD = 0.47 mg cholesterol/g). Streptomyces species COD showed significant changes in activity up to the maximum concentration of 2 U/μM cholesterol used in this investigation (LSD = 0.47 mg cholesterol/g). Surprisingly, even at the low concentration of 0.125 U/μM cholesterol, COD from Pf degraded more than one-third (up to 34.6%) of the egg cholesterol in 2 h. This was the only significantly different reduction between treatments seen at this concentration (LSD = 0.47 mg cholesterol/g). Increasing the concentration of Pf COD further to 1 and 2 U/μM did not produce a reduction in cholesterol that was significantly different (LSD = 0.47 mg cholesterol/g).

Cholesterol degradation was significantly different to that of the control at all concentrations and enzyme types (LSD = 0.41 mg cholesterol/g). The most effective enzyme type/concentration combination was that of Pf COD at 0.5 U/μM.

Cholesterol degradation products.

A number of COD products have been detected in foods including spray-dried egg yolk powder (*19*). The adverse health effects of these oxidation products are still a topic for debate.

In this study, the degradation products of egg cholesterol were examined by TLC and confirmed by MSD. The only steroid-like component detected was 4-cholesten-3-one, for each of the four enzymes. The 4-cholesten-3-one has been described as the first degradation product of cholesterol oxidase catalysis in species of *Nocardia, Brevibacterium, Pseudomonas* and *Streptomyces* by many investigators (*2,5,10,28,31,33*). Aihara et al. (2) also found this compound in egg yolk inoculated with *R. equi* No. 23, a COD producing microorganism, but it was converted rapidly into non-steroid components.

CONCLUSION

The cholesterol degrading capacity of four COD enzymes in egg yolk, a complex food system, has been investigated. All enzymes catalyzed the transformation of cholesterol to 4-cholesten-3-one. More than 50% of egg cholesterol could be reduced by three COD's (Ne, Pf and Ss), within 24 h. The Pf COD was effective over a wide temperature range including at 4°C. The low concentration of COD from Pf required to achieve significant reduction in yolk cholesterol coupled with the ability of this enzyme to degrade cholesterol at 4°C may make this enzyme, com-

pared to the other three investigated, an economical and practical method for lowering the cholesterol level in egg yolk.

ACKNOWLEDGMENTS

We thank the Egg Industry Research Council for financial support, Kym Butler of the Victorian Institute of Animal Science for assistance with the design of our experiments and statistical analysis, Vince Murone of Victoria University of Technology for assistance with GC-MSD analysis, and Harry Laan, post-doctoral fellow from the University of Groningen in the Netherlands, for valuable discussion.

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