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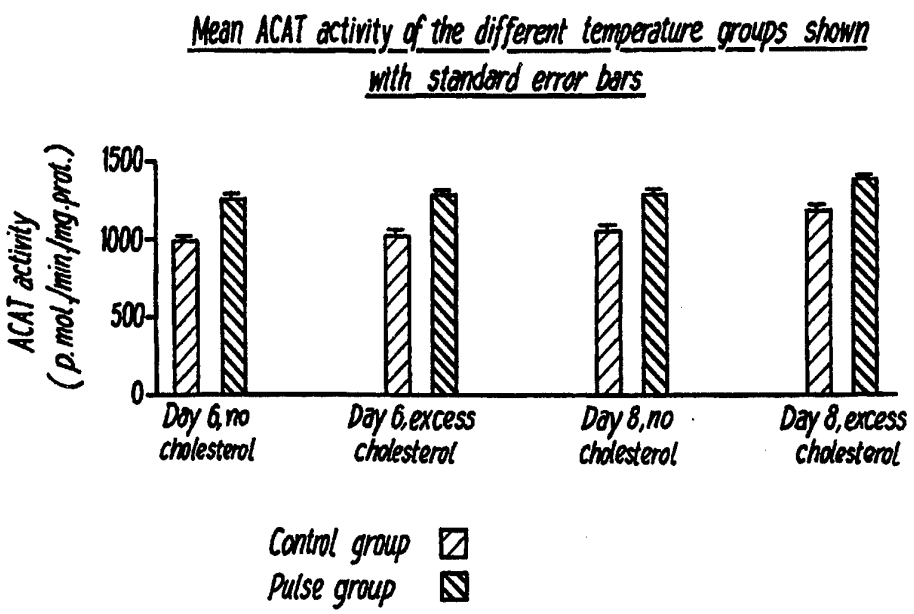

(57) Abstract

A method of influencing the sex of an embryo comprising altering the biologically active amount of acyl co-enzyme A cholesterol acyl transferase (ACAT) in at least part of the embryo. The activity of ACAT may be altered by adding a competitive inhibitor of ACAT, by removing a precursor of ACAT, by manipulating the level of cholesterol in the diet of the mother before laying or by manipulating the levels of cholesterol in an embryo. Genetic material encoding and capable of expressing ACAT can also be used.

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SEX reversion by altering biologically active amount of ACAT

This invention relates to a method of influencing the sex of an embryo.

Sex manipulation of animals is of great commercial importance to the livestock industry. It also has far reaching consequences in the field of medicine.

In the egg producing sector of the poultry industry, slaughter of male chicks at hatching (40 million per annum in the UK) represents a considerable loss of money. The ability to manipulate the sex of embryos to produce females would represent a considerable economic benefit. Similarly in the broiler (meat) section of the poultry industry there is also an advantage to be derived by manipulation of the sex of the chick as male and female are reared separately, reach their slaughter weights at different ages and therefore give differing economic returns.

It has been established for some time that some reptiles (related to avians) eg crocodilians, exhibit a

feature known as temperature-dependent sex determination (TDSD), in which manipulation of the temperature at which the egg is incubated determines the sex of the offspring. Recently, workers at the University of Manchester have devised a temperature incubation regime to enable the sex of the chick embryo to be similarly manipulated (Patent Application No WO 94/13132 the contents of which are fully incorporated herein by reference).

However, whereas in the crocodilians the achievable sex reversal can be complete, ie 100% male or 100% female, in the chick embryo the limit so far achievable by temperature manipulation has been of the order of 20% reversed in favour of the male. In addition, until now the mechanism by which the sex reversal occurred was not understood.

According to the present invention there is provided a method of influencing the sex of an embryo, said method comprising altering the biologically active amount of acyl co-enzyme A cholesterol acyl transferase (ACAT) in at least part of the embryo.

The biologically active amount of ACAT in the embryo may be altered by temperature changes, for example, by increasing or decreasing the temperature of the developing embryo. An alternative method for altering the biologically active amount of ACAT in the embryo is by adding a competitive inhibitor of ACAT, or by sequestering or otherwise removing a precurson of ACAT (such as cholesterol) from the embryo. A typical way of manipulating levels of cholesterol in an embryo would be by adjusting the diet of the mother before laying eggs. The biologically active amount of ACAT may also be altered by phosphorylation or

dephosphorylation of the ACAT molecule as phosphorylation effects ACAT activity. Alternatively, the biologically active amount of ACAT may be dependent upon the fluidity characteristics of a surrounding membrane and agents which affect membrane fluidity may also be used.

The biologically active amount of ACAT present in the embryo can be altered simply by addition of ACAT (optionally in a substantially pure form) or by sequestering ACAT, typically by using antibodies which bind specifically to ACAT. Alternatively, the biologically active amount of ACAT in the embryo may be increased by enhancing synthesis or by decreasing degradation of ACAT within the developing embryo.

ACAT may also be expressed from a recombinant genetic material preferably within the embryo thus producing enhanced levels of ACAT and optionally producing a means of controlling ACAT production by allowing manipulation of ACAT expression. Such recombinant genetic material may include expression control sequences such as promoters and/or enhancers which allow control of the levels of expression of ACAT.

The present invention also provides an embryo having a recombinant DNA sequence containing genetic material encoding for ACAT.

Optionally the embryo is a vertebrate embryo, such as avian, reptilian or amphibian. Alternatively, the embryo may be an invertebrate embryo (such as an insect embryo). A preferred embryo is avian.

The present invention also provides a transgenic animal containing genetic material coding for and able to

express ACAT.

Further, the present invention provides the use of ACAT to manipulate the sex of an embryo.

In another aspect, the present invention provides an animal, the sex of which has been influenced by alteration of the biologically active amount of ACAT present in said animal during embryonic development.

The present invention concerns the identification of an enzyme system intimately associated with the control mechanism known as TDSD demonstrable naturally and fully in crocodilians and most recently successfully applied to the chicken. The methodologies used in the identification of the involvement of this enzyme system were as follows:

1. yolk sac membrane tissue from alligator and chick embryos were obtained throughout the incubation periods of both species. In both increasing levels of ACAT activity were recognised as being specifically involved in cholesterol transfer to the embryo and delivery to the gonads and other tissues. In both species differing levels of ACAT activity were able to be associated with the differential manipulation of the sex of the embryo as achieved via recognised incubation temperature manipulations.

2. tissue fractionation, in particular the preparation of microsomes, for the measurement of ACAT activity was performed by established ultra-centrifugation techniques as outlined by Shand et al (1993).

3. the basis of the determination of ACAT activity in the tissues was the incorporation of radioactivity from ^{3}H-oleoyl CoA into cholesterol ester. The conditions of the tissue microsomal incubation for this measurement was as described by Shand et al (1993). Following incubation the radio-labelled cholesterol oleate ie the measure of ACAT activity, was extracted by established procedures involving solvent extraction techniques and cholesterol ester isolation by thin layer chromatography (Christie, 1982). The radio-labelled cholesterol ester was transferred into scintillation vials and the radioactivity measured by standard scintillation counting procedures. Microsomal protein (used as a base for comparisons of ACAT activity) was determined by standard methodology (Bradford, 1976).

We have now found that in both the crocodilian and the chick systems the effect of temperature on the ultimate sex of the hatchling is accompanied by a major difference in the level and activity of ACAT an enzyme that has at the same time been shown to be essential for the transfer of cholesterol from the yolk to the gonads and other tissues for sex hormone synthesis. In the alligator during the incubation period post 30 days (ie past the period of sexual differentiation) male embryos displayed a level of ACAT that was twice that of females and commensurate with growth rate differences. However, prior to the 30th day of the incubation period ie when TDSD is operational, levels of ACAT in the female embryo were twice that of the male. In the latter case the level of ACAT activity was directly associated with increasing incorporation of cholesterol into the differentiated gonad as opposed to growth. A single experiment with the chicken in

which an increased male population was achieved through incubation temperature manipulation was associated with changes in levels of ACAT. This finding thus provides

a mechanism through which temperature effects are mediated and therefore an alternative means of manipulation or enhancement of incubation temperature regimes on the ultimate sex of the emerged chick.

The following experimental data indicated how ACAT levels in chicken eggs are altered with temperature pulsing.

EXAMPLE

Eggs used in the experiments were Gallus gallus domesticus (ISABROWN) supplier Tom Barrens PLC, Carforth Lancs of known parental flock age (between 28 and 40 weeks) and identical lay date. The eggs were incubated within 48 hours of lay. Eggs were divided into incubation groups, with incubation starting on day 0. The incubation procedure was as follows and according to that described in Patent Publication No WO 94/13132.

In each case eggs were incubated at 38°C 100% humidity with regular turning (Multihatch Automatic Incubator Brinsea Products, UK) unless otherwise stated. Pulsing involved incubating eggs at 38°C with a 36 hour pulse of 22°C, starting on day 3 of incubation.

Samples of approximately 150 eggs were used.

After incubation yolk sacs were removed from the eggs. Different treatment groups were given the following names, and will be referred to by their abbreviated forms:

Control Day 6	C.D6	Yolk sac membranes taken from eggs opened on day 6, incubated at 38°C
Control Day 8	C.D8	Yolk sac membranes taken from eggs opened on day 8, incubated at 38°C
Pulse Day 6	P.D6	Yolk sac membranes taken from eggs opened on day 6 of incubation, which were pulsed for 36 hours, from day 3 at 22°C (otherwise incubated at 38°C
Pulse Day 8	P.D8	Yolk sac membranes taken from eggs opened on day 8 of incubation, which were pulsed for 36 hours, from day 3 at 22°C (otherwise incubated at 38°C)

The pulsing regime is well proven to produce a phenotypic sex ratio of 65:35 males:females (see Patent Publication No WO 94/13132). It was confirmed that conditions of incubation presently used to obtain samples for the investigation consistently produced changes in phenotypic sex in accordance with all results obtained previously and designated in Patent Publication No WO 94/13132 ie 65% males, 35% females. Phenotypic sex was determined by gonadal inspection. Genotypic sex was determined with the use of W chromosome specific probe.

As yolk sac membranes taken from eggs at these early stages of development (days 6 and 8) are very small it was necessary to pool individual membranes from the same treatment group in order to obtain enough tissue to perform the ACAT assay (approximately 2g). Hence the microsomal preparations used are made up of tissue from a number both male and female eggs. All samples were diluted to a standard concentration (7.5 mg

protein/ml microsomal preparation) to allow comparison.

Yolk sac membranes were collected from control and pulse eggs on days 6 and 8 of incubation as indicated by sample names. The membrane was washed in saline solution to remove any yolk, excess moisture was soaked up with blotting tissue. Membranes were then individually wrapped in aluminium foil and dropped in liquid nitrogen to ensure complete inhibition of enzymic reactions. Samples were then stored at - 80°C until use.

Preparation of microsomal fraction

Materials:

Balance, electric homogenizer, ultra centrifuge, glass homogenizer, spectrophotometer.

Reagents:

Bradford Reagent

All other reagents listed in buffers below

Homogenisation Buffer for 500ml pH=7.3

50 mM Tris	3.02 g
50 mM MES	4.88 g
1 mM EDTA	0.22 g
1 mM EGTA	0.19 g
1 mM DTT	0.08 g

Protinase inhibitors:

Antipain, Peptatine, Leupeptide (all conc. 5 mg/ml)

3 x 100µl aliquots

distilled H_2O make volume up to 500 mls

Assay Buffer for 500 ml pH=7.4

50 mM Tris	3.02 g
50 mM MES	4.88 g
distilled H_2O	make volume up to 500 ml

Protocol:

Approx 2g of tissue was suspended in 8 ml of homogenisation buffer and treated as outlined in steps 1-6 below.

1) Homogenise tissue suspension using electric blender;

2) Spin homogenised sample for 15 minutes at 10,000 G at 4°C;

3) Spin supernatant for 30 minutes at 100,000 G at 4°C

4) Discard supernatant and wash pellet in Homogenisation buffer and spin again 30 minutes at 100,000 G at 4°C;

5) Discard supernatant and resuspend pellet in Assay buffer; and

6) Measure protein concentration using Bradford Reagent and spectrophotometer. Store at -80°C until needed.

Liposome Preparation for AcylCo-A Cholesterol Transferase (ACAT) Assay

Liposomes were used in the ACAT assay to provide an excess of cholesterol. ACAT activity was measured with and without excess cholesterol in order to show whether changes found in ACAT activity were due to increased availability of cholesterol or to some other factor

linked to the change in incubation conditions.

Samples with no added cholesterol are indicated by the sign (-), those assayed with excess cholesterol are denoted (+).

Materials:

Amicon ultrafiltration cell.
Diaflow membrane (YM-100) stored in 20% alcohol.
Heater block.
Magnetic stirrer

Reagents:

Cholesterol	10 mg/ml in ethanol
Phosphatidyl choline	46 mg/ml in ethanol
Phosphate Buffer	0.1 M KH_2PO_4

Protocol:

1) Add 20 ml of warmed Phosphate buffer in amicon ultra filtration cell with membrane (YM-100 Diaflow) on magnetic stirrer;

2) Mix 1:1 molar ration of cholesterol; phosphatidyl choline (300µl of each);

3) Inject 500 µl of the cholesterol/choline mix into the warm Phosphate buffer in cell;

4) Allow mix to filter down to approximately 5 ml under 10 lb/inch pressure;

5) Wash residue in further 20 ml warm phosphate buffer and filter down to approximately 2 ml; and

6) Discard filtrate and dilute liquid residues (Liposome Preparation) in 6 ml of phosphate buffer

N.B. Liposome Preparation can be kept at ambient temperature for up to two weeks.

Cholesterol Estimation of Liposome Preparation

It was necessary to estimate the amount of cholesterol in the liposome preparation to ensure that the addition of liposomes to the assay mix will provide cholesterol in excess. A saturation curve was calibrated for ACAT, by assaying the enzyme but with differing concentrations of the liposome preparation as indicated in Figure 1. The enzyme reaches saturation for concentrations greater than and equal to 158 μg cholesterol/ml.

A standard cholesterol solution of 15 μg/ml (dissolved in ethanol), is needed to calibrate the fluorimeter.

Standard Buffer/Liposome concentrations:

Standard	200μl standard cholesterol solution + 2ml buffer (A)
Blank	2.2 ml buffer (A)
Liposome	200 μl liposome preparation + 2 ml buffer (A) (in duplicate)

Protocol:

1) 50μl microsome + 950μl MeOH:$H_2$0.3:2 + 1 ml CH_3Cl:MeOH.2:1

2) Blend and spin at 4000 G in bench top centrifuge for 2 minutes;

3) Remove lower layer into clean glass scintillation vial;

4) Add 100 µl distilled water and 1000 µl CH_3Cl:MeOH/2:1 to original tubes blend and spin again;

5) Take lower layer and pool with initial extraction in scintillation vial;

6) Concentrate sample under N_2;

7) Heat at 110°C for 20 minutes;

8) Redissolve in 2 ml EtOH;

9) Add 200 µl of above solution of 2 ml (+) and (-) buffer;

10) Incubate at 37°C in shaking water bath for 30 minutes; and

11) Measure luminescence of samples in fluorimeter.

Cholesterol Estimation of Microsome Preparations

The cholesterol content of all the microsome preparation assayed for ACAT activity was estimated to enable the comparison of ACAT activity to the free cholesterol content of the microsome preparation, and also to the amount of cholesterol ester present.

Reagents:

Methanol:Water / 3:2
Chloroform: Methanol / 2:1
Phosphate Buffer (pH 7.4) 0.1M KH_2PO_4
Cholesterin oxidase
Cholesterol peroxidase
Cholesterol esterase
Triton X-100
Sodium Cholate

Para-hydroxyphenol acetic acid (PHP-A)
Cholesterol

Two buffers were prepared, with (+) and without (-) cholesterol esterase, the amount of buffer was dependent upon the number of samples tested with 4 ml, of each buffer per sample. The buffers contained the following reagents in the following ratios:

	Concentration of Reagents	**No esterase (-)**	**With esterase (+)**
Phosphate buffer	0.1 M	21.1 ml	16.9 ml
Triton X-100*	0.5 %	2.1 ml	2.1 ml
Sodium Cholate*	20 mM	2.1 ml	2.1 ml
PHP-A*	4 mg/ml	6.3 ml	6.3 ml
Cholesterol oxidase	187.9 µl oxidase + 8.21 ml phosphate buffer	4.2 ml	4.2 ml
Cholesterin esterase	92.3 µl esterase + 3.26 ml phosphate buffer	-	4.2 ml
Cholesterol peroxidase (1 mg/ml)	478.6 µl peroxidase + 7.91 ml phosphate buffer	4.2 ml	4.2 ml

*All dilutions and solutions were made up with 0.1 M phosphate buffer.

A standard cholesterol solution of 15 µg/ml (dissolved

in ethanol), was used for calibration of the fluorimeter.

Acyl Co-A Cholesterol Acetyl Transferase (ACAT) Assay

Reagents:

^{3}H-oleoyl CoA
Co enzyme A
^{14}C Cholesteryl 1-^{14}C oleate
Cholesterol oleate "cold"
MeOH/H_2O 3:2
Chloroform/Methanol 2:1
Bovine serum albumin
Dithiothreitol (DTT/Cleands reagent)

Method:

Two buffers were prepared with (+) and without (-) excess cholesterol (provided by the liposomes). The table below shows the proportions of reagents needed in these buffers.

	40 mM Cleands Regent DTT	60 mg/ml Bovine Serum Albumin BSA	0.1 M KH_2PO_4 pH 7.4	Liposome Preparation
Buffer (no cholesterol)	181 µl	228 µl	2684 µl	-
Buffer (excess cholesterol)	181 µl	228 µl	864 µl	1268 µl

Preparation of ^{14}C cholesteryl oleate (internal standard):

70 mg "cold" cholesterol oleate + 100 µl (5µCi/ml ^{14}C cholesteryl oleate) + 15 ml $CHCl_3$/MeOH 2:1.

Preparation of ^{3}H-oleoyl CoA (substrate)

[according to the method of Goldman, P and Vagelos, P.R. (1961)]

Remove solvent from radioactive fatty acid and, if necessary, add sufficient 'cold' oleic acid (3.54 μmol/mg or 3.17 μmol/μl) to bring the specific activity up to the above value. Re-dissolve in 1 ml dichloromethane. Add a 20% molar excess of dry (ie re-distilled and stored over KOH pellets) triethanolamine (7.55 μmol/μl) and an equal molar excess of dry (ie re-distilled) ethylchloroformate (11.07 μmol/μl). Mix and leave in fridge for about 10 minutes. Blow off reagents and add a 50% molar excess of coenzyme A (1.29 μmol/mg) in 2 ml tert-butanol: 0.1M $NaHCO_3$ (2:1). Check pH and adjust to 8.0 - 8.5 using conc $NaHCO_3$. Leave at ambient temperature for 30 minutes. Check with sodium nitroprusside solution filter paper. A slightly pink coloration is obtained which is greatly increased by methanolic NaOH (indicates most free sulphydryl groups used up by reaction but exposed again on hydrolysis by strong alkali). Acidify to a pH less than 1 with 5M H_2SO_4 and then add 5ml ice cold 1M perchloric acid and leave for at least 5 minutes. Spin and pour off supernatant. Add a further 5ml cold 1M PCA, re-suspend pellet and spin again. Wash pellet twice with 5ml acetone and twice with 5ml diethyl ether. Dry off under N_2. Re-dissolve in H_2O pH to 4.0-4.5 with 1M NaOH and make up to about 2ml.

Count (ie usually 10 μl to ml then count 10 μl of this solution) and work out yield from this figure and the original specific activity. Store in aliquots at -70°C in sufficient volume to give 10 aliquots of the diluted substrate below.

Dilute with 0.1M KH_2PO_4 (pH = 7.4) and add cold oleoyl CoA to give ACAT assay substrate containing 1 μCi and 20 nmol in 10 μl. Store at -80°C until use.

Dilution of microsome preparations:

Comparisons of ACAT activity between different groups of samples were made following dilution of all microsomal preparations to a standard concentration of 7.5 mg protein/ml microsomal preparation.

Protocol:

1) 20 μl of microsome preparation (conc. 7.5 mg/ml) + 170 μl (+) and (-) buffer in individual glass tubes;

2) Pre-incubated in shaker/water bath at 30°C for 30 minutes;

3) Add 10 μl H-oleoyl CoA substrate at 15 second intervals allow mixture to react for 5 minutes;

4) STOP reaction with 1.8 ml MeOH/H_2O 3:2;

5) To each tube add: 50 μl ^{14}C cholesteryl oleate standard and 2 ml CH_2Cl/MeOH 2:1 and mix with whirl mixer; and

6) Remove lower layer into scintillation vial and concentrate sample under N_2 for application to thin layer chromatoplates.

Thin layer Chromatography and Scintillation counting

Reagents:

Glacial acetic acid

Scintillation fluid (emulsifier safe)

Kiesel gel 60G

Hexane

Ether
Formic Acid
Dichlorofluoroscein

Method:

Plates:

Clean plates were coated with an even layer (0.5mm thick) of Kieselgel 60G; 50g Kieselgel 60G and 100 mls distilled water was sufficient to make 5 plates

Chromatography:

Separation of the liquids were performed using hexane: diethylether: formic acid in ratio 95:5:1 by volume.

Protocol:

1) Redissolve concentrated sample in 250 μl CH_3Cl/MeOH 2:1;

2) Spot onto TLC plate;

3) Run plate till completion;

4) Dry plate in air and spray with dicholofluoroscein;

5) Identify band under UV light and scrape into clean scintillation vials;

6) To each vial add 400 μl distilled water, 50 μl acetic acid, 8 ml scintillation fluid; and

7) Mix contents of vials well and count in liquid scintillationcounter in $^3H/^{14}C$ mode.

All reagents used were of the highest grade possible and obtained from reputable suppliers.

The results show the following:

1. The distribution of ACAT activities of the yolk sac membranes of the different groups, specifically comparing the distributions of the temperature pulsed groups with respect to their relative control group.

2. The relationship between microsomal ACAT activity and the free cholesterol content.

3. The relationship between cholesterol ester content of microsome preparations and corresponding ACAT activity.

Calculation of ACAT activity and its distribution in the various treatment groups

The specific activity of ACAT was calculated from the ratios produced by the scintillation counter using the following formula, where the $^{3}H/^{14}C$ ratio for the individual samples is the average of the duplicate samples counted.

A = ACAT activity of sample, measured in p.mol./min/mg.prot

$$A = B \times C$$

where

B = $[(^{3}H/^{14}C \text{ sample}) - (^{3}H/^{14}C \text{ blank})]$

$$C = \frac{\text{Standard dpm } 2 \times 1000 \times 1000 \times 20 \text{m.mol substrate}}{\text{Substrate dpm } 1 \times 5 \text{ minutes} \times 150 \ (\mu\text{g protein/assay})}$$

Table Mean ACAT activities of the different treatment groups and their standard deviations

	C6(-)	c6(+)	P6(-)	P6(+)	C8(-)	C8(+)	P8(-)	P8(+)
Mean ACAT activity pmol/min/ mg.prot	992.0	1018.0	1263.3	1284.4	1053.1	1185.0	1289.5	1379.5
Standard Deviation	395	221	311	329	257	434	313	339

ACAT activity is in units of p.mol./min/mg.prot

These results are illustrated on in Figure 2.

All treatment groups have normal distributions, therefore 2-tailed non-paired T-tests were used to test for significance between the pulse and control groups.

	C6(-):P6(-)	C6(+):P6(+)	C8(-):P(-)	C8(+):P(+)
Level of Significance	P<0.05	P<0.08	P<0.05	P<0.06

The relationship between microsomal cholesterol ester content and ACAT activity

Fluorimeter readings for the microsomal preparations incubated with (+) buffer, ie with cholesterin esterase are used to calculate total cholesterol. The cholesterol ester content of each preparation is then simply TOTAL CHOLESTEROL-FREE CHOLESTEROL.

To calculate Total cholesterol content:

$$\text{total cholesterol} = \frac{\text{Fluorimeter reading} + 0.92}{76.59}$$

Where values (0.92) and (76.59) are obtained from the calibration curve for total cholesterol which has the following linear equation:

$$y = 76.59X - 0.92$$

Figures 3 and 4 illustrate microsomal cholesterol ester plotted against ACAT activity for each of the day 6 and day 8 samples respectively.

The relationship between microsomal ACAT activity and free cholesterol content.

Graphs 5 and 6 show ACAT activity plotted against free microsomal cholesterol content. The free cholesterol content, and indeed the cholesterol ester content of microsomal preparations was calculated directly from the fluorimeter readings of cholesterol content using the following formulae:

1 Fluorimeter readings from sample incubated with (-) buffer, ie buffer without cholesterol esterase are used to calculate free cholesterol.

$$\text{Free cholesterol} = \frac{\text{Fluorimeter reading} - 2.975}{115.89}$$

Values (2.975) and (115.89) are derived from the calibration curve for free cholesterol which has been previously calculated to have the linear equation

$$y = 115.89X + 2.975$$

CONCLUSIONS AND INTERPRETATION

The experimentation and results described herein indicate that

1. temperature pulsing increases ACAT activity and that the increase in activity is highly significant (P) 0.05.

2. data on the relationship between ACAT activity and free microsomal cholesterol is linear.

3. data on the relationship between ACAT activity and microsomal cholesterol ester content is linear.

This means that ACAT is involved in immobilisation of cholesterol from the yolk (maternal sources) into the embryo. Cholesterol esters are the precursors of the sex steroid (oestrogens/androgens) biosynthetic pathways. This demonstrated that increasing ACAT increases cholesterol ester supply, increases androgen biosynthesis and hence increases the percentage males - the effects seen with temperature pulsing. The converse would also apply to increase percentage females - decreasing ACAT levels by limiting material sources of cholesterol, or supplying competitive inhibitors of ACAT or removing precursors of ACAT would increase percentage females

There is therefore an implication that a mechanism according to the present invention or temperature pulse action can be used to manipulate the percentage males and females in a livestock industry.

It appears that supplementing yolk (either by feeding the mother or by injection to the yolk) with

cholesterol (or cholesterol esters) may increase the percentage males without temperature pulse.

Also, supplementing yolk by dietary manipulation of females or by injection to the yolk of competitors of cholesterol for example a sterol of plant origin, may decrease the percentage males or increase the percentage females without the temperature pulse.

Supplementation of the above with the appropriate temperature pulse, could synergise to increase further the alteration of sex ratio.

Oleic acid is a fatty acid which is specifically involved with ACAT. By increasing or reducing oleic acid levels, enzyme activity of ACAT may be altered, thereby altering the sex ratio distortion. Fatty acid compositions could be altered by dietary means.

Thus, it appears that any of the following combinations;

fatty acid manipulations and temperature pulse;

fatty acid manipulations and temperature pulse and cholesterol supplements or competitors; and

fatty acid manipulations and cholesterol supplements or competitors

may synergistically alter the sex ratios.

ACAT activity which appears to be a measure of the ability to transfer yolk cholesterol into the embryo of both the alligator and chick and therefore to the tissues but in particular to the gonads was altered to

a high degree by the temperature of incubation.

It is also apparent that ACAT activity alters between male and female embryos depending upon whether the changes were responding to the period of TDSD or growth.

This feature was identifiable and correlatable in both species to the change of sex able to be achieved by the incubation temperature regime through TDSD.

The activity of ACAT can be altered by manipulation of lipid parameters within the yolk.

The identification of the relationship between ACAT activity and alteration of the sex ratio and gonadal changes presents a means whereby sex reversal in the embryo may be achieved or TDSD may be enhanced by dietary or other simple means.

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CLAIMS

1. A method of influencing the sex of an embryo comprising altering the biologically active amount of acyl co-enzyme A cholesterol acyl transferase (ACAT) in at least part of the embryo.

2. A method as claimed in Claim 1 wherein the amount of ACAT is altered by temperature changes.

3. A method as claimed in Claim 1 or 2 wherein the amount of ACAT is altered by adding a competitive inhibitor of ACAT to the embryo to produce females.

4. A method as claimed in Claim 1 or 2 wherein the amount of ACAT is altered by sequestering or otherwise removing a precursor of ACAT from the embryo to produce females.

5. A method as claimed in any of the preceding Claims wherein, the amount of ACAT is altered by manipulating the diet of the mother.

6. A method as claimed in any of the preceding Claims wherein the amount of ACAT is altered by manipulating levels of cholesterol in an embryo.

7. A method as claimed in Claim 1 wherein ACAT is expressed from a recombinant genetic material.

8. A recombinant nucleotide sequence containing genetic material encoding ACAT and capable of expressing ACAT.

9. An embryo containing a nucleotide sequence as claimed in Claim 8.

10. A transgenic animal containing genetic material coding for ACAT.

11. The use of ACAT to manipulate the sex of an embryo.

12. An animal, the sex of which has been influenced by alteration of the biologically active amount of ACAT present in said animal during embryonic development.

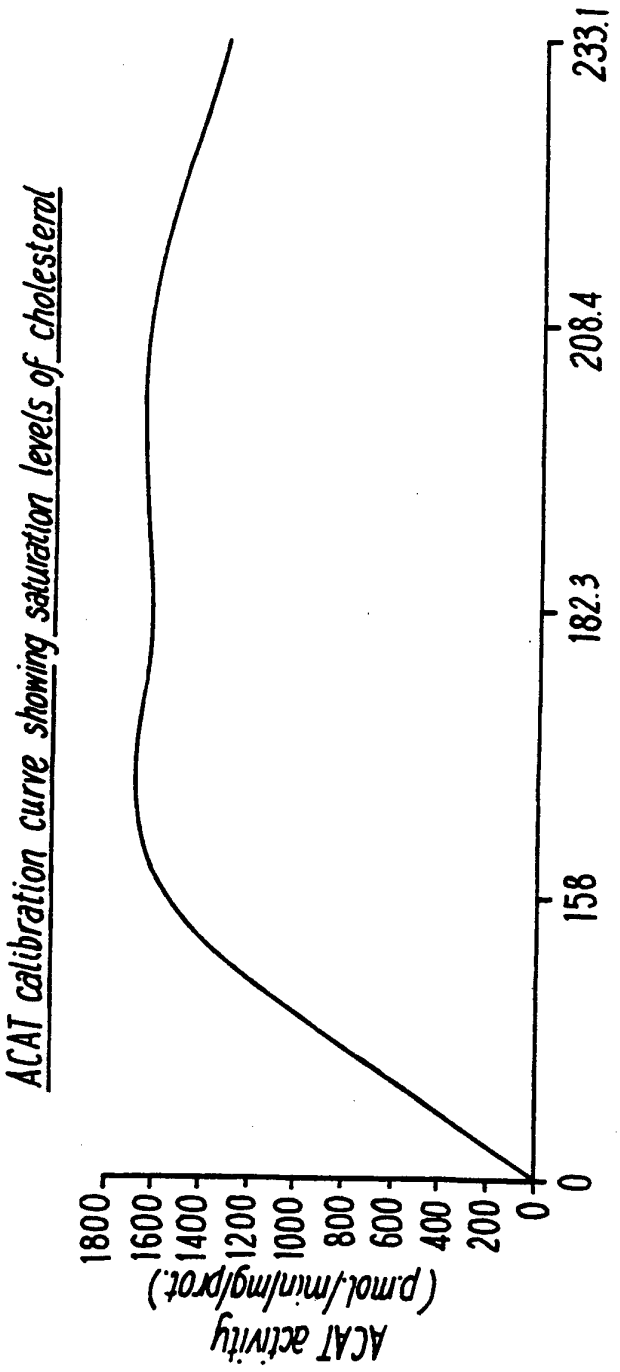
ACAT calibration curve showing saturation levels of cholesterol
ACAT activity
(p.mol./min/mg/prot.)
1800
1600
1400
1200
1000
800
600
400
200
0
0
158
182.3
208.4
233.1
Cholesterol Concentration (µg/ml)

Fig. 1

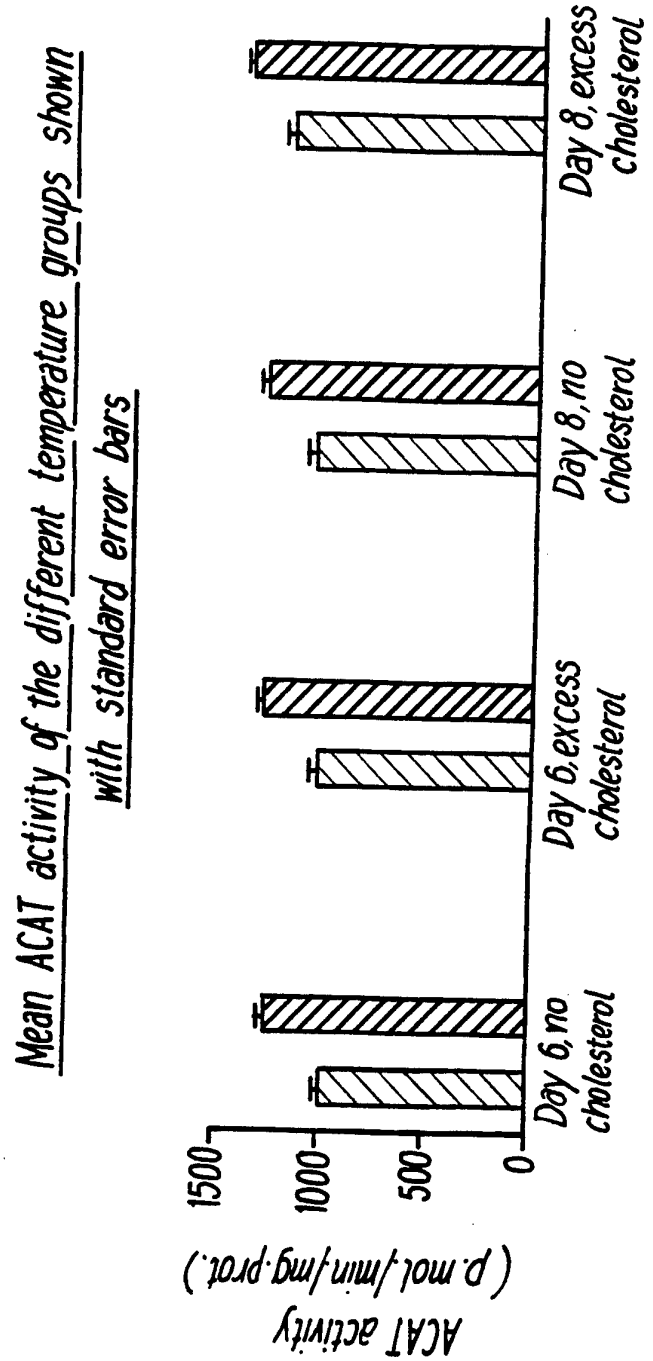
Mean ACAT activity of the different temperature groups shown with standard error bars
ACAT activity
(P. mol./min/mg.prot.)
1500
1000
500
0
Day 6, no cholesterol
Day 6, excess cholesterol
Day 8, no cholesterol
Day 8, excess cholesterol
Control group
Pulse group

Fig. 2

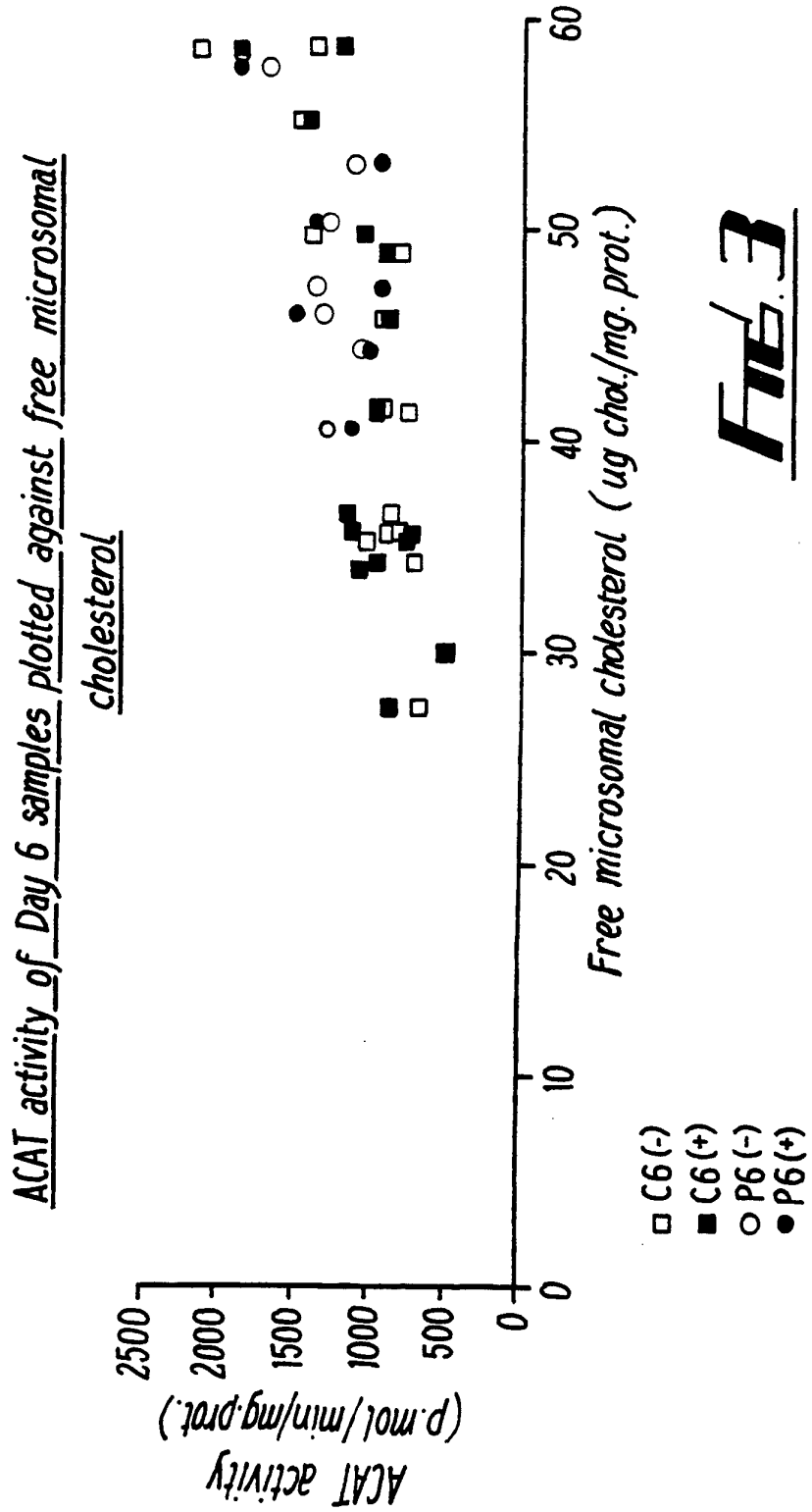
ACAT activity of Day 6 samples plotted against free microsomal cholesterol
ACAT activity (p.mol/min/mg.prot.)
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
Free microsomal cholesterol (ug chol./mg. prot.)
C6(-)
C6(+)
P6(-)
P6(+)

Fig. 3

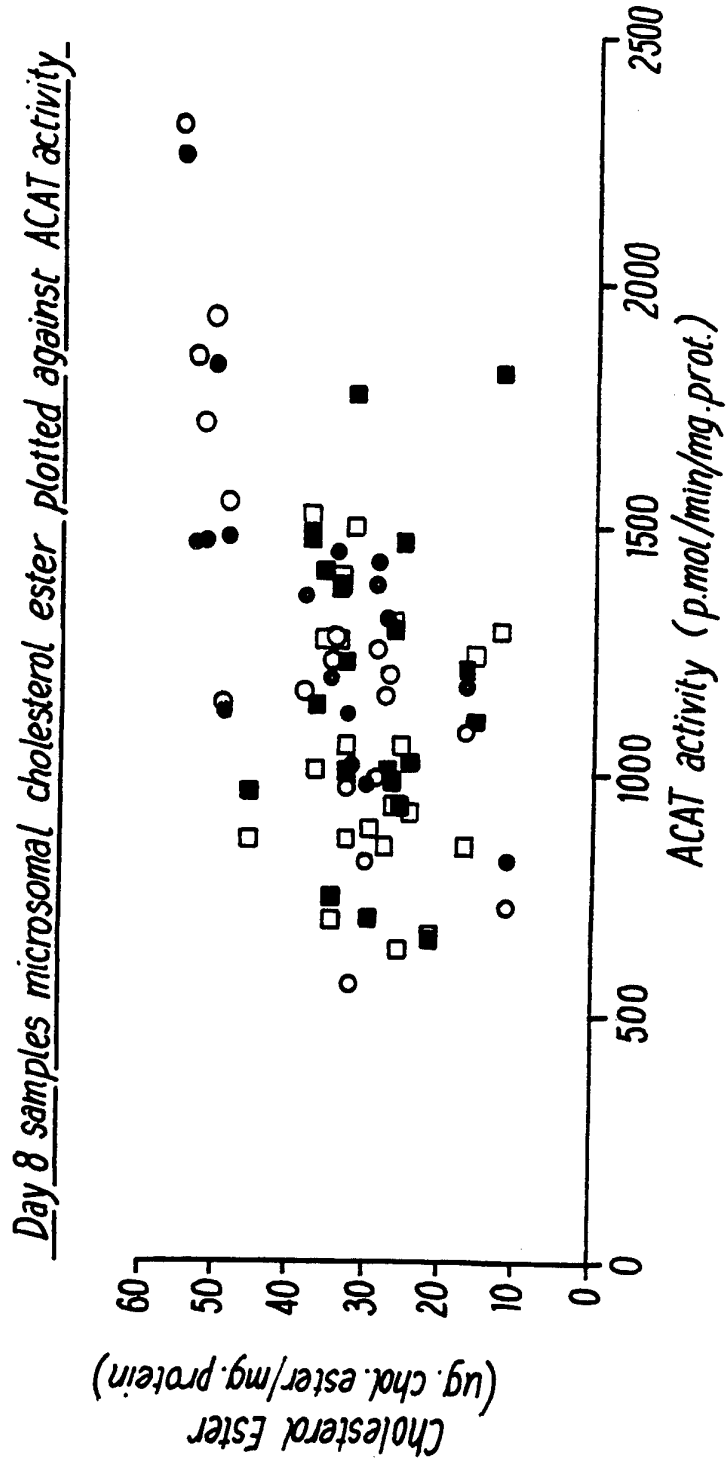
Day 8 samples microsomal cholesterol ester plotted against ACAT activity
Cholesterol Ester (ug. chol. ester/mg. protein)
ACAT activity (p.mol/min/mg.prot.)
0
10
20
30
40
50
60
500
1000
1500
2000
2500
□ C8 (-) C.E.
■ C8 (+) C.E.
○ P8 (-) C.E.
● P8 (+) C.E.

Fig. 4

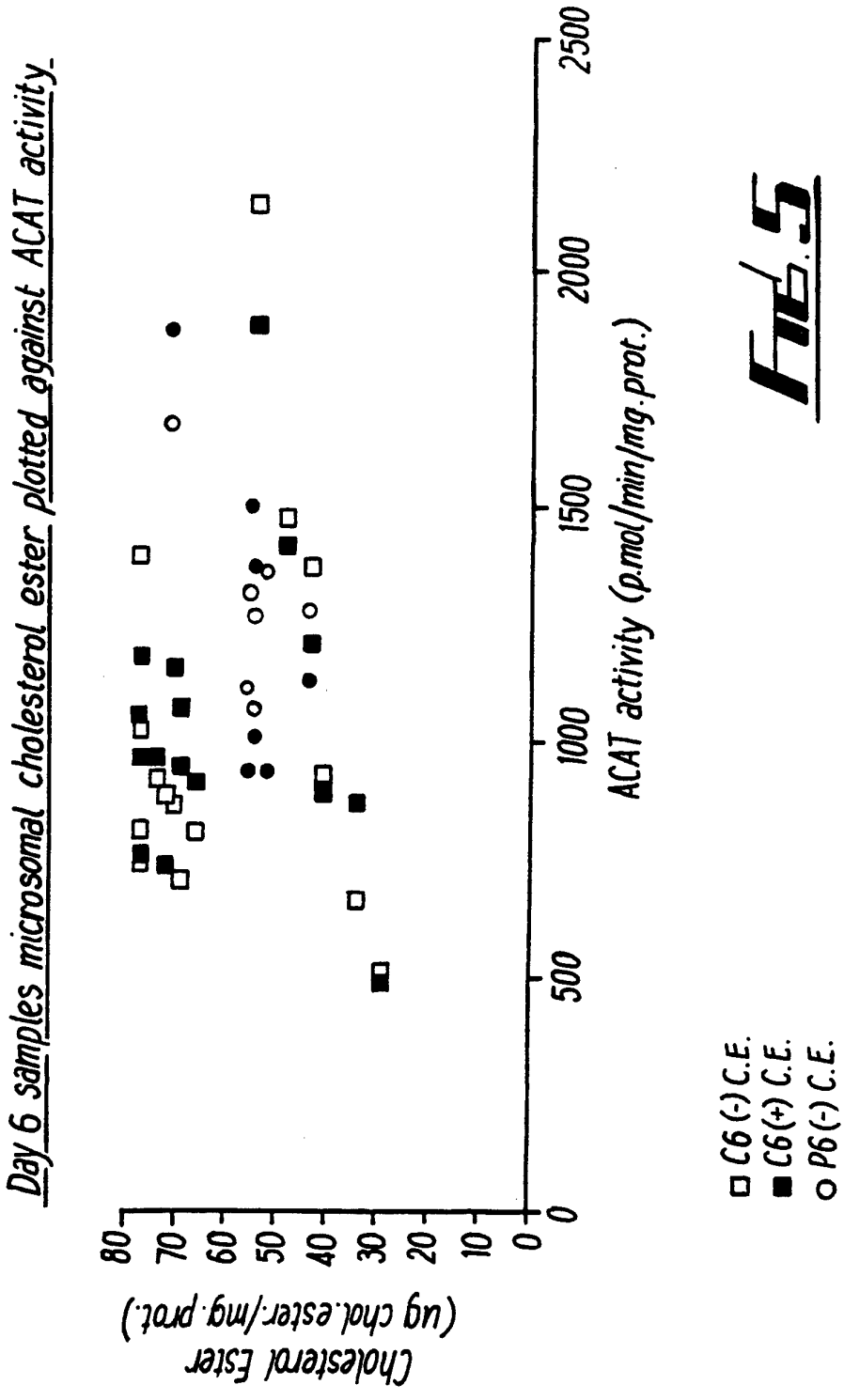
Day 6 samples microsomal cholesterol ester plotted against ACAT activity.
Cholesterol Ester (ug chol.ester/mg.prot.)
ACAT activity (p.mol/min/mg.prot.)
0
10
20
30
40
50
60
70
80
500
1000
1500
2000
2500
C6 (-) C.E.
C6 (+) C.E.
P6 (-) C.E.
P6 (+) C.E.

Fig. 5

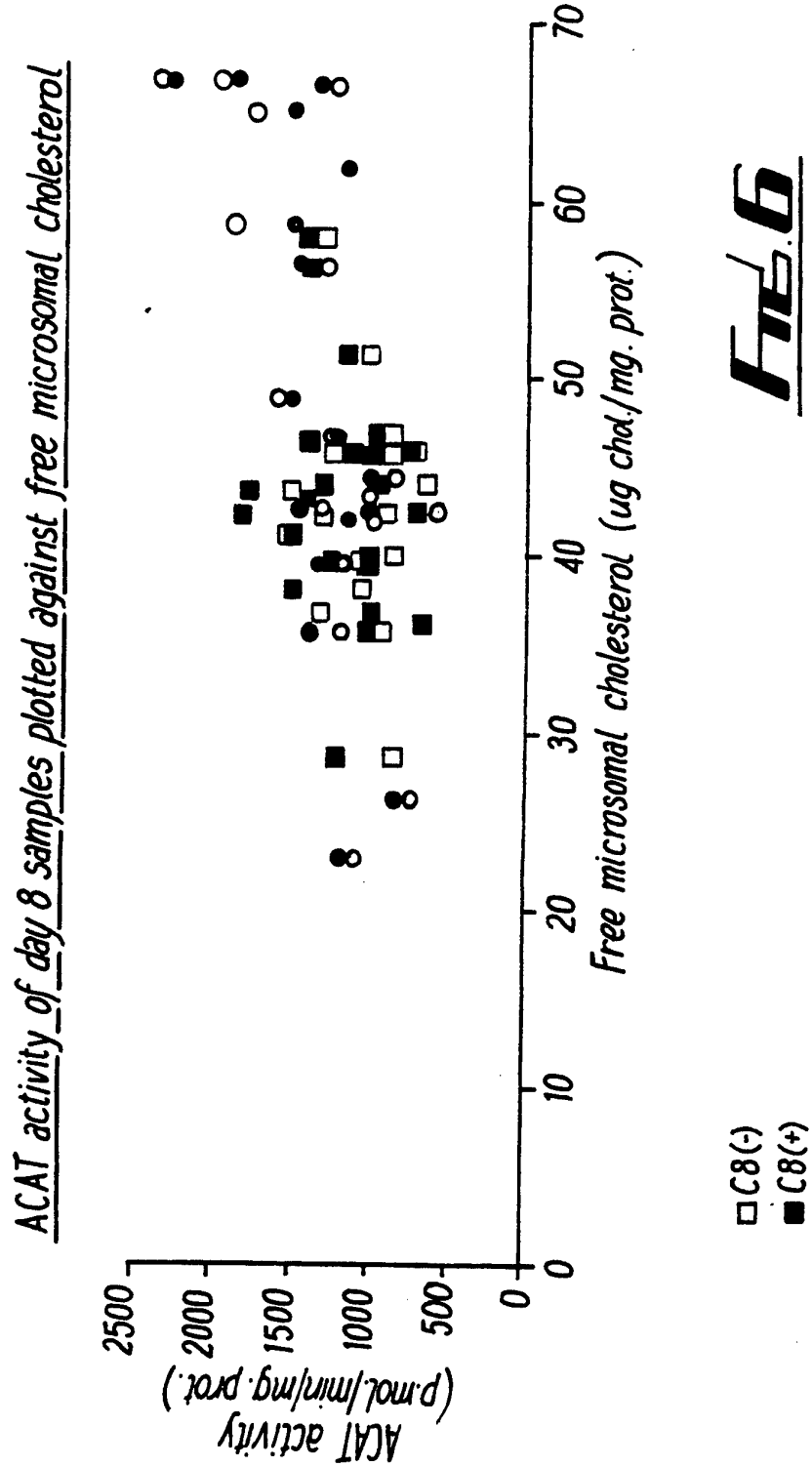
ACAT activity of day 8 samples plotted against free microsomal cholesterol
ACAT activity (p.mol./min/mg. prot.)
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
70
Free microsomal cholesterol (ug chol./mg. prot.)
C8(-)
C8(+)
P8(-)
P8(+)

FIG. 6

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/GB 95/01517

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C12N15/00 A61D19/00 A01K67/027 //A61K38/45, C12N9/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 13132 (BRITISH TECHNOLOGY GROUP LIMITED) 23 June 1994 cited in the application see the whole document ---	2,12
A	EP,A,0 433 084 (MERCK CO. INC.) 19 June 1991 see the whole document ---	1
P,A	BIOCHIMICA ET BIOPHYSICA ACTA, vol.1213, no.2, 14 July 1994 pages 224 - 230 SHAND, J.H. ET AL. 'The esterification of cholesterol in the liver of the chick embryo' see the whole document ---	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

4

Date of the actual completion of the international search	Date of mailing of the international search report
4 September 1995	08.09.95

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOCHEMICAL SOCIETY TRANSACTIONS, vol.20, no.4, 1992 page 340S SHAND, J.H. ET AL. 'Acyl Coa Cholesterol Acyltransferase activity in the yolk sac membrane of the chick embryo' see the whole document & 643rd Meeting of the biochemical Society Coventry, UK July 22-23 1992	1
A	LIPIDS, vol.24, no.2, 1989 pages 105 - 108 BRUSCALUPI, G. ET AL. 'Cholesterol metabolism in frog Rana esculenta liver seasonal and sex-related variations' see the whole document	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. ıal Application No
PCT/GB 95/01517

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9413132	23-06-94	CA-A-	2151608	23-06-94
		GB-A-	2273431	22-06-94
EP-A-0433084	19-06-91	AU-B-	640721	02-09-93
		AU-A-	6813190	20-06-91
		JP-A-	4211315	03-08-92