Effect of Oestradiol 3-Benzoyl on the Concentrations of Retinal and Lipids in Cod Plasma

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1. Determinations of retinal, total lipid and lipid phosphorus were made on 10 ml. samples of cod plasma. 2. Immature control fish, injected with 0.2 ml. of carrier oil/kg. wt., had $0.73 \pm 0.12 \mu\text{g.}$ of retinal/100 ml. of plasma, and maturing male fish had similar concentrations. Maturing female fish had about $10 \mu\text{g.}$ of retinal/100 ml. of plasma. 3. Immature male or female cod given single intramuscular injections of 1 mg. of oestradiol-17 β 3-benzoyl in 0.2 ml. of oil/kg. wt. had $8.54 \pm 0.59 \mu\text{g.}$ of retinal/100 ml. of plasma after 5 days and about $25 \mu\text{g.}/100 \text{ ml.}$ of plasma after 10 days. 4. Oestradiol injections had little effect on the concentration of plasma phospholipids, and no effect on lipids other than phospholipids. 5. For all 116 fish examined, regardless of sex or treatment, the concentration of plasma phospholipid was significantly correlated with that of lipids other than phospholipids ($r = 0.727$), and phospholipids formed 50.4% of the total lipids in cod plasma. 6. Alcohol dehydrogenase was purified from cod liver and shown to oxidize retinol to retinal. It was completely inhibited by 0.1 mM-oestradiol. Alternative modes of action of oestradiol are discussed.

Retinal (vitamin A₁ aldehyde) is present in the eggs of fish, amphibians, reptiles and birds, and in many species of marine teleosts, including cod, it is the predominant form of vitamin A in the eggs (Plack & Kon, 1961; Plack, Woodhead & Woodhead, 1961). The concentration of retinal in the ovaries of cod increases during the period of yolk deposition in the eggs and falls to zero after spawning (Plack *et al.* 1961). Corresponding to this increase in concentration in the ovaries, the concentration of retinal in the blood plasma of mature female cod increases some sixfold, and falls to a low value after spawning, whereas the concentration in the plasma of mature males and of juvenile male and female cod remains at a low value throughout the year (Plack & Woodhead, 1966).

These findings suggest that the retinal in the ovaries is produced in some other tissue and carried to the eggs by the blood, and that the mobilization or synthesis of retinal may be controlled by the female sex hormones. Gottfried, Hunt, Simpson & Wright (1962) have found low concentrations of oestradiol-17 β and of oestrone in mature ova of cod, but there is as yet no proof that these mammalian hormones are the active ones in fish. Nevertheless, a number of workers have shown that injections of oestradiol into fish produce changes in the concentrations of calcium, protein and lipid in

the blood similar to those found in females with developing ova (Bailey, 1957; Ho & Vanstone, 1961; Urist & Schjeide, 1960-61; Fleming, Stanley & Meier, 1964). In the work reported in the present paper, injections of oestradiol benzoate into immature male or female cod were found to produce increases in the concentration of retinal in the plasma, the values reached being similar to, or greater than, those found in female fish with developing ovaries.

MATERIALS AND METHODS

Fish. Cod (*Gadus morhua* L.) were caught in shallow water in Aberdeen Bay by seine net and transferred to tanks of aerated sea water at 10-14°. They were not fed during the experiments. This stock of fish spawns in March-April, and cod were taken during the period November-April. The sex and maturity of the fish cannot be distinguished by external appearance, except immediately before spawning, but, as a rough guide to maturity, fish weighing less than 2500 g. are likely to be immature juveniles, that is not yet producing eggs or sperm each year, whereas those over 2500 g. are more likely to be sexually mature. When the experiments were completed and the fish killed, the sex was determined after dissection and the maturity estimated from the maturation coefficient ($100 \times \text{wt. of gonad/wt. of whole fish}$).

The maturation coefficient increases with development of the gonad and can reach 23% in ripe fish of both sexes

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(Sorokin, 1957, 1960). In studying the effect of oestradiol, results for fish with a maturation coefficient of 0.6% or less have been selected, i.e. sexually immature fish or, with early samples, a few sexually mature fish in which development of the gonads had not yet begun. Initial results indicated that males and females gave similar responses to oestradiol, and mean values given are for fish of both sexes. Since the suitability of individuals could not be accurately determined until after the experiments were completed, the number of fish/group is variable.

Chemicals. M.S.222 (*m*-ethoxycarbonylanilinium methanesulphonate) was obtained from Sandoz Products Ltd. (London, W. 1). Oestradiol-17 β 3-benzoate dissolved in arachis oil-ethyl oleate (4:1, v/v) (Oestroform; British Drug Houses Ltd., London, N. 1) was used for injection as supplied, at concentrations of 5mg./ml. or 1mg./ml., or diluted with sterile solvent. Heparin injection B.P. (Pularin; Evans Medical Ltd., Speke, Liverpool) was diluted to 2500 i.u./ml. with water before use. Diethyl ether and SbCl₃ were purified as described by Plack & Kon (1961). Al₂O₃, for chromatographic adsorption analysis (British Drug Houses Ltd.), was heated to 150° for 2 hr. before use. Crystalline retinal was obtained from Kodak Ltd. (Kirkby, Liverpool).

For the enzyme experiments, oestradiol-17 β 3-benzoate and NAD⁺ from Sigma (London) Chemical Co. Ltd. (London, S.W. 6), crystalline retinol from Roche Products Ltd. (Welwyn Garden City, Herts.) and Tween 40 (polyoxyethylene sorbitan monopalmitate) from Honeywell and Stein Ltd. (London, W. 1) were used. Light petroleum (b.p. 40–60°), *n*-hexane and ethanol were redistilled before use for the extraction and chromatographic separation of retinal and retinol from the reaction mixture. Other chemicals were of A.R. grade, or the nearest available equivalent.

Treatment of fish. Fish were anaesthetized in sea water containing about 1 part of M.S.222 in 10000 and weighed, and the injection was made into the epaxial muscle behind the head. The dose of oestradiol was based on the weight of the fish, and the volume injected was usually 0.2 ml./kg. wt. Control fish received a similar injection of sterile solvent (arachis oil-ethyl oleate, 4:1, v/v). The fish were allowed to recover in an isolated tank of aerated sea water and then transferred to the holding tank.

At the end of the experimental period, the fish were killed by a blow on the head and dried to remove sea water, and their tails were cut off. Blood from the caudal artery was collected in a beaker previously rinsed with heparin solution. The fish were weighed again, and the gonads were removed and weighed to give the maturation coefficient. Samples of ovaries were fixed in Bouin's fluid for histological examination in cases of doubtful maturity.

Extraction of lipid. Plasma was separated from the blood samples by centrifuging, and 10 ml. was blended with 225 ml. of CHCl₃-methanol (2:1, v/v); the mixture was filtered and washed, and the solvent was removed as described by Plack, Miller & Ward (1966). The residual lipid was dissolved in CHCl₃ and the volume made up to 20 ml.

Determination of retinal. Al₂O₃ was weakened by the addition, with shaking, of 10% (w/w) of water to the dry powder, and a column (25 cm. $\times$ 0.4 cm. diam.) was prepared from a slurry in light petroleum (b.p. 40–60°). The CHCl₃ was removed *in vacuo* from 4 ml. of the lipid extract, 0.5 ml. of *n*-hexane was added to dissolve the residue and the whole

was transferred quantitatively to the Al₂O₃ column. The column was developed with a gradient of diethyl ether in light petroleum, produced by feeding diethyl ether-light petroleum (1:1, v/v) into 75 ml. of light petroleum contained in a mixing vessel, from which the column was supplied. Retinyl esters and some lipids were eluted in the first 8 ml., which was discarded. The next 12 ml. of eluate contained the retinal; its volume was reduced to about 1 ml. in a stream of N₂, the whole was transferred quantitatively to a 1 cm. glass spectrophotometer cell, and the remainder of the solvent was removed under N₂. To the residue, 0.1 ml. of CHCl₃ was added, followed by 0.4 ml. of 14% (w/v) SbCl₃ in CHCl₃. The maximum extinctions reached at 665 m μ and at 840 m μ were read with a Unicam SP.500 spectrophotometer fitted with a pin-hole and lens, the cell being raised in the holder so that the light-beam passed through the 0.5 ml. of liquid.

For each series of determinations, standards were run with crystalline retinal. A solution in *n*-hexane was prepared of about 5 μ g./ml., the precise concentration being determined from the extinction at 370 m μ and $E_{1\text{cm}}^{1\%}$. 1700 at this wavelength. A sample (0.1 ml.) was transferred to a spectrophotometer cell, the solvent removed with N₂, and 0.1 ml. of CHCl₃ followed by 0.4 ml. of SbCl₃ solution added for readings as above. In the calculation of $E_{1\text{cm}}^{1\%}$ for the Carr-Price test with pure retinal, and of the concentration of retinal in the sample, the extinction at 840 m μ was subtracted from that at 665 m μ (see Plack & Kon, 1961). The precision ($\pm 2 \times \text{s.d.}$) of the colorimetric test was $\pm 0.009 \mu$ g. for 0.05 μ g. samples of retinal and $\pm 0.022 \mu$ g. for 0.40 μ g. samples.

Dehydroretinal is also present in cod plasma (Plack & Woodhead, 1966), but on the micro scale employed here no attempt has been made to determine both retinal and dehydroretinal. The extinction at 665 m μ in the Carr-Price test represents the maximum extinction due to retinal together with 0.38 times the maximum extinction due to dehydroretinal at its λ_{max} , 740 m μ . The recovery of retinal added at the extraction step was 85%.

Determination of lipid and lipid phosphorus. The solvent was removed from 4 ml. and 2 ml. samples of each lipid extract under N₂ at 60°, and total lipid determined by weighing. Samples of 1.0 ml., 0.5 ml. and 0.25 ml. of each extract were used for determination of phosphorus by the method of Allen (1940). Phospholipid concentrations were obtained by multiplying the lipid phosphorus values by 25, and lipids other than phospholipids by subtracting the phospholipid value from the total lipid concentration.

Purification of cod liver alcohol dehydrogenase (alcohol-NAD oxidoreductase, EC 1.1.1.1). Cod liver was blended with 20 vol. of acetone at 4° and an acetone-dried powder prepared. Acetone extracts most of the lipid (present to the extent of 65% in some livers), retinyl esters and retinol. The acetone-dried powder, 10–20 g., was extracted with 14 times its weight of water for 2 hr. at room temperature and centrifuged for 30 min. at 5000g. The supernatant liquid was brought rapidly to 52°, maintained at this temperature for 5 min., rapidly cooled to room temperature and centrifuged for 1 hr. at 5000g. Solid (NH₄)₂SO₄ was added to the supernatant liquid, the pH being held at 6.5 by the addition of aq. NH₃, and the precipitate formed between 40% and 60% saturation was collected by centrifuging for 1 hr. at 5000g, dissolved in 10 mM-sodium phosphate buffer, pH 7.0, and dialysed overnight at 4° against 10 mM-sodium phos-

phate buffer, pH 7.0. The dialysed solution was diluted with 10 mM-sodium phosphate buffer to give a protein concentration of about 20 mg./ml., and $(\text{NH}_4)_2\text{SO}_4$ precipitation was carried out again, the material precipitating at 40%, 50% and 60% saturation being collected by centrifuging for 1 hr. at 5000g, dissolved in 10 ml. of buffer as before, and dialysed overnight against the same pH 7.0 buffer. The enzyme and protein contents of the preparations were measured as described by Bonnichsen & Brink (1955) at 25° with a Unicam SP.800 spectrophotometer, the enzyme, buffer and NAD^+ being preincubated until no further increase in extinction occurred at 340 $\text{m}\mu$ before addition of ethanol. Enzyme was present in both the 40–50%-saturated and the 50–60%-saturated $(\text{NH}_4)_2\text{SO}_4$ precipitates, the purities being 0.5% and 1.2% respectively; the latter fraction was used for most of the experiments.

Retinol (5 mg.) and Tween 40 (0.1 ml.) were dissolved in 1 ml. of CHCl_3 , the solvent was removed *in vacuo* and 4–9 ml. of hot water was added to give an aqueous dispersion of retinol. The rate of oxidation of retinol to retinal was followed at 400 $\text{m}\mu$ with 2.3 ml. of 0.1 M-sodium phosphate, 0.1 M-sodium pyrophosphate, or 0.1 M-glycine-NaOH buffers at pH values from 7.0 to 10.5. 0.1 ml. of NAD^+ (10 mg./ml.), 0.2 ml. of enzyme solution and 0.1 ml. of retinol dispersion (added last after 30 min. preincubation), giving final concentrations of 0.105 M-retinol and 0.075% (v/v) Tween 40. At 400 $\text{m}\mu$, the extinction due to retinol and NADH is negligible and the increase in extinction is due to the formation of retinal. Oestradiol was added before preincubation as 10 μl . of an acetone solution to give final concentrations of 0.1 mM–0.1 μM with 0.1 M-sodium phosphate buffer, pH 7.0.

Proof of the formation of retinal was provided by the following reaction mixtures: (a) 3.8 ml. of 0.1 M-sodium phosphate buffer, pH 7.0, 0.8 ml. of enzyme solution [40–50% $(\text{NH}_4)_2\text{SO}_4$ ppt.] and 0.2 ml. of retinol dispersion; (b), as (a) but with boiled enzyme; (c) and (d), as (a) and (b) but with 3.8 ml. of 0.1 M-sodium pyrophosphate buffer, pH 9.0, in place of phosphate buffer. The retinol dispersion was added after 30 min. preincubation and the mixtures were left for a further 1 hr. at room temperature in the dark. Ethanol (5 ml.) was then added to each of the four mixtures, which were extracted by shaking with three 20 ml. lots of light petroleum. The residues obtained on removing the solvent were dissolved in 3 ml. of *n*-hexane and transferred to 7 cm. $\times$ 1.3 cm. diam. columns made from a slurry of Al_2O_3 in 8% (v/v) ethanol in *n*-hexane. Ethanol in *n*-hexane (1%, v/v) (30 ml.) eluted retinal, characterized by its absorption curves in ethanol solution and in the Carr-Price test, and 30 ml. of 8% (v/v) ethanol in *n*-hexane eluted unchanged retinol.

Statistical methods. Mean values are given with their standard errors. Student's *t* test was used to determine the significance of differences between groups.

RESULTS

Retinal. The concentrations of retinal in the plasma of control fish receiving an injection of the carrier oil, 0.2 ml./kg. wt., and killed 5 days after injection, are shown in Fig. 1. Immature fish of maturation coefficient $< 0.6\%$ had low concentrations, similar in males and females, the mean for seven fish being $0.73 \pm 0.12 \mu\text{g}$. of retinal/100 ml. of

plasma. As the ovaries increased in weight and yolk was deposited in the ova, the concentration of retinal increased to the order of $10 \mu\text{g}$./100 ml. of plasma but, with male fish, increase in maturation coefficient did not result in an increase in concentration of plasma retinal.

Nine cod of maturation coefficient $< 0.6\%$ that received an injection of oestradiol benzoate (1 mg./kg. wt.) had a mean retinal concentration of $8.54 \pm 0.59 \mu\text{g}$./100 ml. of plasma 5 days after injection, 11 times the concentration in the control fish. Fig. 2 shows the results at different times after the injection of 1 mg. of oestradiol benzoate/kg. wt. The marked rise in the concentration of plasma retinal began 3 days after injection and did not reach a maximum within 10 days. There was one high value at 1 day after injection, which could be due to injection into, or close to, a blood vessel in the muscle, since histological examination revealed no yolk in the ova.

Variation of the dose of oestradiol benzoate gave the results shown in Fig. 3, and with doses of 0.5, 1.0 and 2.0 mg./kg. wt. there was no significant difference in the concentration of plasma retinal produced in 5 days. Two low doses of oestradiol benzoate were tested over 10 days; with 100 μg . in 0.2 ml. of carrier/kg. wt., six fish had a mean concentration of $5.42 \pm 0.83 \mu\text{g}$. of retinal/100 ml. of plasma, and with 10 μg . of oestradiol benzoate in 0.2 ml. of carrier/kg. wt., seven fish had a mean concentration of $0.59 \pm 0.04 \mu\text{g}$. of retinal/100 ml. of plasma, similar to that for control fish receiving the carrier only.

Phospholipids. There was no significant difference between male and female cod in the mean concentration of phospholipid in the plasma, either within groups or for all fish. The mean concentration for female fish receiving an injection of 0.2 ml. of oil/kg. wt. and of maturation coefficient $< 0.6\%$ was not significantly different from that for similar fish of maturation coefficient $> 1.0\%$, and the same was true for male cod, so that maturity did not seem to affect the concentration of plasma phospholipid.

The mean value for seven control fish was $0.723 \pm 0.085 \text{ g}$./100 ml. of plasma, and in only two groups was this significantly increased by oestradiol treatment. Two fish receiving 1 mg. of oestradiol benzoate/kg. wt. and killed after 10 days each had a phospholipid concentration of 1.12 g./100 ml. of plasma, and eight fish injected with 2 mg. of oestradiol benzoate/kg. wt. and killed after 5 days had $0.987 \pm 0.049 \text{ g}$./100 ml. of plasma. The mean value for all 116 fish examined was $0.835 \pm 0.020 \text{ g}$./100 ml. of plasma.

Lipids other than phospholipids. There was no significant difference in the plasma concentrations of lipids other than phospholipids between sexes,

Fig. 1. Concentrations of retinal in the plasma of cod of differing maturity injected intramuscularly with 0.2 ml. of arachis oil-ethyl oleate (4:1, v/v)/kg. wt. ○, Female; □, male.

Fig. 2. Concentrations of retinal in the plasma of cod (maturation coefficient <0.6%) given an intramuscular injection of 1 mg. of oestradiol benzoate/kg. wt. and bled at different times after injection. ○, Female; □, male; ●, mean values with their standard errors for two female and three male fish at 0 days (no injection) and for five female and four male fish at 5 days.

Fig. 3. Mean values with their standard errors for the concentration of retinal in the plasma of cod (maturation coefficient <0.6%, male and female combined) given various intramuscular injections of oestradiol benzoate and bled 5 days after injection. The zero value is the mean for seven fish injected with carrier oil only; 0.1 mg./kg., two fish; 0.2 mg./kg., two fish; 0.5 mg./kg., nine fish; 1.0 mg./kg., nine fish; 2.0 mg./kg., eight fish.

between immature fish and mature fish with developing gonads, between oestradiol-treated and control fish, or between fish receiving an injection of carrier oil (with or without oestradiol benzoate) and fish receiving no injection. The mean value for all 116 fish examined was 0.821 ± 0.020 g./100 ml. of plasma.

Proportion of phospholipids. The concentration of phospholipid in the plasma was significantly correlated with the concentration of lipids other than phospholipids. For all 116 cod examined, regardless of treatment, $r = 0.727$, and phospholipids represented 50.4% of the total lipids.

Cod liver alcohol dehydrogenase. The enzyme purified from cod liver had optimum pH 9.7 with ethanol as substrate and pH 8.9 with retinol. The oxidation of retinol to retinal was shown by extraction of reaction mixtures (a)–(d) (see the Materials and Methods section). Mixture (a), pH 7.0, gave 28 μ g. of retinal, with $\lambda_{\max}$ 377 m μ in ethanol and 663 m μ in the Carr–Price test, whereas mixture (b) gave no retinal. The retinol oxidized, given by the difference between the retinol concentration in mixture (b) and that in mixture (a), was 38 μ g. The corresponding values for (c) and (d), pH 9.0, were 62 μ g. of retinal extracted and 95 μ g. of retinol oxidized, the discrepancies being due to the combination of some retinal with protein as a Schiff's base.

The effect of oestradiol on the oxidation of retinol was studied at pH 7.0. In the absence of oestradiol, the rate of increase in extinction at 400 m μ was proportional to the amount of enzyme used. Addition of oestradiol at a final concentration of 0.1 mM completely inhibited the reaction, the inhibition decreasing with the oestradiol concentration, with 50% inhibition at 2 μ M- and no inhibition at 0.1 μ M-oestradiol. There was no evidence of activation of the enzyme at lower concentrations of oestradiol.

DISCUSSION

The method used here for the determination of retinal is the same as that reported previously (Plack *et al.* 1966), but scaled down so that the lipid extract from 2 ml. of plasma can be used. In the final colorimetric reaction, E_{665} 0.010 corresponds to about 0.6 μ g. of retinal/100 ml. of plasma.

The values for the concentration of plasma retinal in female cod at the beginning of maturation supplement the results of Plack & Woodhead (1966) for Arctic cod, when no samples were obtained between the end of October and January. One fish appears out of line in these results, with 3.5 μ g. of retinal/100 ml. of plasma and a maturation coefficient of only 0.70%, but histological examination showed the maturing eggs to be about one-quarter full of yolk.

Parkes (1937) has shown that single intramuscular injections of 1 mg. of oestradiol 3-benzoate in oil give a prolonged oestrogen action in capons lasting 9 days, whereas the free hormone has only a transient effect; the ester has therefore been used in these experiments. Administration of oestrogens alone appears to affect the mobilization or synthesis of substances required for deposition in the eggs, and, since in the absence of gonadotrophins there is probably no uptake by the eggs, the concentrations in the serum can be raised to values higher than those found in normal fish with developing eggs. The concentrations of plasma retinal found here

after immature male or female cod were injected with oestradiol are, in general, similar to the concentrations found normally in maturing female cod, but the value 10 days after an injection of 1 mg. of oestradiol benzoate/kg. wt. show that higher than normal concentrations can be obtained.

There are no previous reports of the effects of oestrogen injections on plasma retinal, but Gardiner, Phillips, Maw & Common (1952) found a 40-fold increase in the concentration of retinyl esters in the plasma of oestrogenized immature pullets. Preliminary experiments with cod showed that the concentrations of retinyl esters and retinol in the plasma were not increased by oestradiol injections.

The absence of significant increases in the concentration of lipids in the plasma after oestradiol treatment, with the exception of slight increases of phospholipid with a high and a prolonged dose, is unexpected, since other workers, with different fish, have found a positive response (Bailey, 1957; Ho & Vanstone, 1961; Urist & Schjeide, 1960–61). With normal Arctic cod, Plack & Woodhead (1966) found a small increase in concentration of total lipids in the plasma of female fish during maturation of the ovaries, and mature females had higher concentrations than mature males. The high and relatively constant proportion of phospholipids in the total plasma lipids may have some bearing on the transport of lipid in cod.

In addition to its large stores of retinyl esters, cod liver contains about 1 μ g. of retinal/g. wet wt. (P. A. Plack & C. M. Ward, unpublished work), and this amount would be sufficient to account for the plasma concentrations found after oestradiol injections. However, the time lag of 3 days before significant increases are found in the plasma suggests that more than a simple release from stored retinal is involved. Activation of alcohol dehydrogenase, resulting in the oxidation of retinol to retinal, would also be a rapid process, and experiments with the enzyme show it to be inhibited, rather than activated, by oestradiol, at least *in vitro*. Oestradiol may affect the concentration of retinal in plasma by synthesis of new enzyme, or by synthesis of a carrier protein, and it may be that retinal is transported by the egg phosphoprotein complex, the concentration of which is increased by oestradiol injection (Bailey, 1957; Ho & Vanstone, 1961; Urist & Schjeide, 1960–61).

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