THE ROLE OF CALCITONIN IN THE CONTROL OF CALCIUM METABOLISM OF THE CHICK EMBRYO

by

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ABSTRACT

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The literature relevant to calcium homeostasis in the chick embryo and the mature bird is reviewed. Evidence is presented to show that a high circulatory level of calcitonin, as measured by a specific radioimmunoassay occurs during the late stages of incubation in chick embryos. A mild hypercalcaemia persists at this time but is unlikely to be the stimulus for the release of calcitonin. The β-agonist isoprenaline stimulates and the β-blocker propranolol inhibits the release of calcitonin, suggesting that a β-adrenergic stimulatory pathway exists in the chick embryo. Hypoxia, which increases until 'pipping' (a physiological stage related to respiratory function), but is relieved at this time, may be the activator of the pathway, particularly since the calcitonin levels fall precipitously after pipping. However, the caltitonin peak did not follow pipping when this stage was artificially advanced or retarded.

In chick embryos exogenous calcitonin failed to alter the plasma concentrations of calcium, magnesium, phosphate, sodium or potassium, nor did it affect blood or extracellular fluid volume, although it did have a transient effect upon the rate of disappearance of ^{45}Ca and $^{2}PO_4$ from the plssma. Calcitonin restricted the degree of hypercalcaemia when this was artificially induced by intravenous injection of calcium. Removal of circulating calcitonin by the use of specific antibodies resulted in hypercalcaemia, suggesting that the calcitonin released naturally in chick embryos functions to restrict a hypercalcaemic stimulus.

A restrictive function for calcitonin was also found in young cockerels and pullets given high calcium diets and in laying hens when plasma calcium was not being utilised for egg-shell calcification.

Tracer studies with ^{45}Ca demonstrated the importance of the yolk-sac in embryonic calcium homeostasis since the proportion of ^{45}Ca found in the yolk-sac decreases towards the end of incubation, at which time the yolk calcium was utilised for skeletal calcification.

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CHAPTER 1

REVIEW OF THE LITERATURE

Introduction

Since the discovery of calcitonin (CT) (Copp, Davidson and Cheney, 1961; Copp, Cameron, Cheney, Davidson and Henze, 1962) its role in calcium homeostasis and its interaction with parathyroid hormone (PTH) and vitamin D metabolites have been much studied. In mammals the emphasis has moved from a control system involving only changes in PTH levels to one in which the antagonistic action of CT may be of some importance, particularly as a fine control mechanism. Hirsch et al (1963 and 1964) demonstrated the thyroid origin of the new hormone and Pearse (1966) showed that the parafollicular cells of the thyroid, which he renamed C-cells, were responsible for the secretion of CT. Pearse and Cavalheira (1967) were the first to demonstrate the ultimobranchial (UB) origin of the C-cells and their migration to the thyroid in mammals. The presence of C-cells in the UB glands of all lower vertebrates so far studied which possess them indicated "an important and hitherto unrecognised function for the UB glands" (Copp, Cockcroft and Kueh, 1967).

In the higher vertebrates it is now established that the major action of CT is the inhibition of PTH/vit D induced bone resorption (Wallach, Chausmer, Mittleman and Dimish, 1967), and it is not surprising therefore that CT has its greatest action in young animals since they have a much more rapid turnover of their skeletal system. Other actions of CT have not been excluded and will be discussed in this review.

In birds the function of CT is by no means clear. The evidence available suggests that whilst CT is secreted in response to a hypercalcaemic challenge its ability to reduce plasma calcium concentration below normal is questioned. During the egg-laying cycle the total plasma calcium concentration rises some 2-3 fold, although most of this increase is associated with the yolk protein vitellogenin and changes in the physiologically important ionic calcium may be small. The resorption of skeletal calcium by the laying bird to supply the large amounts of calcium necessary for egg-shell formation represents a severe strain upon calcium homeostasis and CT may be involved in the control of plasma calcium concentration and/or the degree of bone resorption. Changes in the concentration of gonadal hormones further complicate calcium homeostasis at this time.

The role, if any, of CT in the chick embryo is a new avenue of research and many of the experiments presented in this thesis have sought to define a role for CT in the maintenance of plasma calcium concentration in the embryo at a time when the transport of calcium from the shell to the developing skeleton may well strain the normal homeostatic mechanisms.

Development of the Chick Embryo

This section will be restricted to the development of the chick embryo with respect to its calcium metabolism. The utilisation of the egg-shell as a source of skeletal calcium by the developing chick embryo was the subject of much controversy and is well reviewed by Needham (1931). Work since this time has left no doubt that about 80% or more, (about 100 mg), of the calcium content of a newly-hatched chick is derived from the inner surface of the eggshell, the remainder coming from the albumen and yolk.

In a notable paper, Johnston and Comar (1955) examined the distribution and contribution of calcium from the albumen, yolk and shell by injecting 25 μCi ^{45}Ca into the albumen of fertile chicken eggs prior to incubation. They found that the embryonic ash increased exponentially between 3 and 9 days and between 9 and 19 days and that the calcium content increased exponentially between 3 and 9 days, between 9 and 15 days, and between 15 and 21 days at ever increasing rates. The embryonic ^{45}Ca derived from the albumen (which freely exchanged with ^{40}Ca of the inner shell layer) ranged from 0.0008% of the dose at 3 days to 82% at 20 days. Of particular interest in this experiment was the use of specific activity measurements which indicated that up to about 10 days, most of the embryonic calcium was derived from the yolk. After 10 days, the shell contributed large amounts of calcium to both the embryo and the yolk. A similar experiment was performed by Nozaki, Horri and Takei (1954) with similar results.

The movement of calcium from the shell to the embryo involves first the solubilisation of the calcium carbonate of the shell and thence its transport to the embryonic circulation via the chorio-allantoic (C-A) membrane. There were two theories of shell resorption. The first suggested that respiratory CO_2 leads to a solution of the eggshell as calcium bicarbonate and was crudely tested by bubbling CO_2 through an eggshell which contained water or albumen (Buckner, Martin and Peter, 1925).

This certainly resulted in the solubilising of some calcium but the quantity of CO_2 required was many times greater than that formed by the embryo (Mankin, 1929) although the bicarbonate content of blood in the C-A circulation does increase throughout incubation (Glaser and Piehler, 1934). The second theory is that an acid (possibly citric acid) is the factor secreted by the C-A which results in shell resorption and indeed the levels of citric acid in the embryo do increase progressively throughout incubation. However, there is no other evidence to suggest this theory. Whatever this other acid may be its effect is diminished by incubating eggs in an atmosphere containing 9% CO_2 (Crooks and Simkiss, 1974). The results of the latter authors strongly argue against the respiratory CO_2 theory of shell resorption since if this were correct it would be reasonable to assume that hypercapnic incubation conditions would enhance shell resorption, not inhibit it.

As well as its respiratory function the C-A membrane has been extensively studied for its calcium transporting role. The differentiation of the C-A cells is not complete until day 14 of incubation which corresponds to the onset of rapid calcium transport (Leeson and Leeson, 1963; Stewart and Terepka, 1969). The calcium transport properties have been studied in vitro (Terepka, Stewart and Merkel, 1969; Terepka, Coleman, Garrison and Spataro, 1971; Moriarty, 1968; Moriarty and Terepka, 1969; Garrison and Terepka 1972a and 1972b; Moriarty, 1973) using isolated C-A membrane mounted in an Ussing type chamber and in vivo (Crooks and Simkiss, 1975; Kyriakides and Simkiss, 1975) using a perspex annulus attached to the C-A membrane in the region of the air sac. In general it can be concluded that calcium transport by the C-A membrane is an active process requiring oxidative phosphorylation and that the calcium transported across the cells is compartmentalised. The involvement of calcium specific ATP-ase, sensitive to sulphydryl binding agents (e.g. iodoacetate), has also been suggested, particularly at the site of the initial calcium uptake. The calculated rates of entry of calcium in the in vivo experiments agree closely with the observed rate of calcium uptake in intact eggs. A diagrammatic representation of a developing chick embryo is shown in Fig. 1.1.

The Ultimobranchial (UB) Gland

The UB gland has been shown to contain CT in a number of different species. Le Dourain and Le Lièvre (1970) clearly showed that the cells

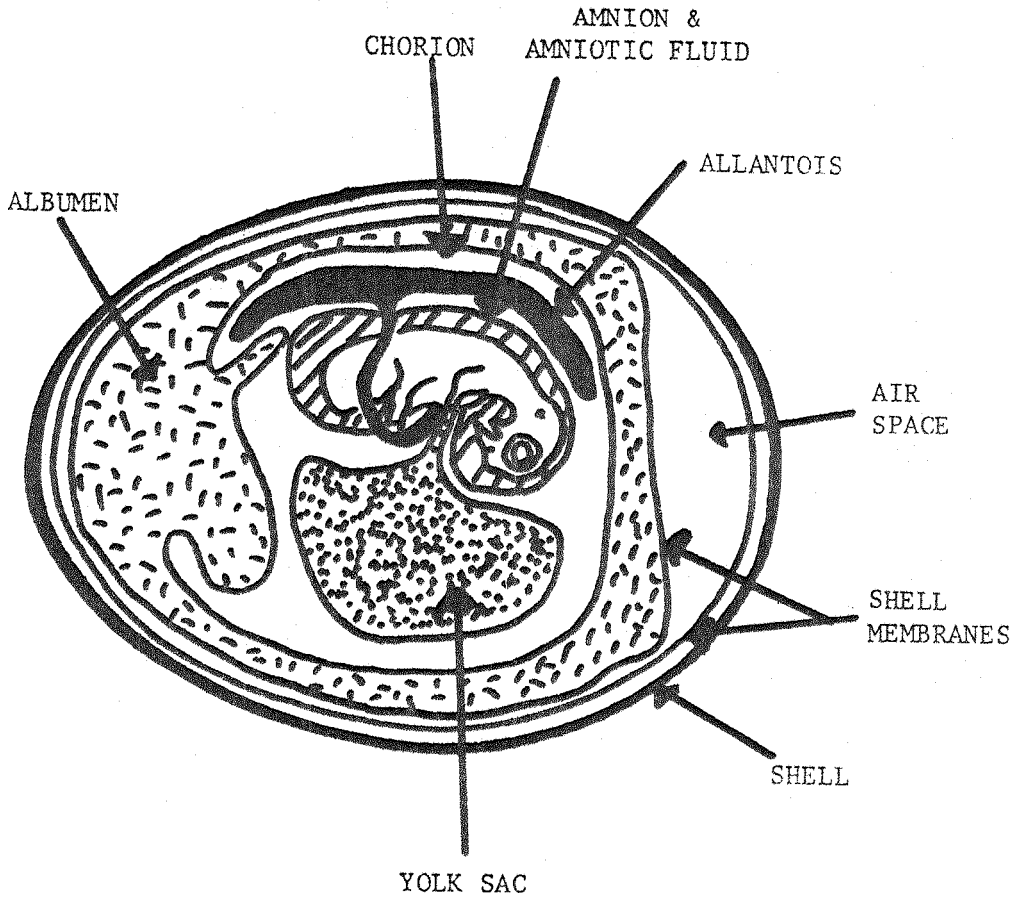


FIG. 1.1 Diagrammatic representation of a developing chick embryo in the early stages of incubation

responsible for the synthesis of CT arose in the neural crest and were of ectodermal origin and that these cells migrate in mammals to the thyroid whereas in birds and fish this migration is complete and the distinct UB gland is thus formed. However there is some evidence to suggest that, even in the lower vertebrates some C-cells remain associated with the thyroid (Pearse and Carvalheira, 1967) and parathyroid glands (Feinblatt , Tai and Kenny, 1975). The presence of CT secreting cells in the parathyroids of chickens is of particular interest and should be borne in mind whenever experiments are quoted in which chickens have been UBX and thus assumed to be free of a source of CT. Clearly this may be a false assumption and the accessory CT-secreting cells may be sufficient to maintain some supply of CT.

The UB glands of birds are not encapsulated and have a diffuse appearance. Both their gross anatomy and their location within the bird vary considerably and depend upon strain, age and indeed the individual. They are to be found in the area bounded by the carotid and subclavian arteries and are more easily recognised in the freshly killed bird where they appear pink due to their relatively rich blood supply.

An excellent review of the anatomy, structure and histology of the UB gland has been given by Hodges (1967). The ultrastructural observations of Stoeckel and Porte (1967, 1969 and 1970) and the earlier work of Nonidez (1935) drew attention to the well developed nerve supply from the vagus. Smaller nerve bundles also enter the glands from the recurrent nerve and sympathetic system (Dudley, 1942). Large bundles (about 60 μ in diameter) of myelinated fibres enter the glands and divide into smaller bundles (12-20 μ) which spread throughout the glands and the smallest branches of these enter into direct contact with the secretory cells but no definite synapses have been observed.

Chemistry and Biosynthesis of Calcitonin

The complete amino-acid sequence of porcine (Potts, Niall, Keutmann, Brewer and Deftos, 1968; Bell, Barg, Colucci, Davies, Dziobkowski, Englert, Heyder, Paul and Snedsker, 1968), bovine (Brewer and Ronan, 1969), ovine (Potts, Niall, Keutmann and Le Quin, 1972), human (Neher, Riniker, Rittel and Zuber, 1968; Neher, Riniker, Zuber, Ritter and Kahnt, 1968), salmon (Niall, Keutmann, Copp and Potts, 1969) and eel (Otani, Noda, Yamauchi, Watanabe, Matsuda, Orimo and Narita, 1975) calcitonins have been determined and the chemical synthesis of each has

been carried out (Guttmann, Pless, Sandrin, Jaquesnoud, Bossert and Willems, 1968; Sieber, Riniker, Brugger, Kamber and Rittel, 1970). In all cases the molecule consists of 32 amino acids, of which only 9 positions are homologous. The common features include a 1-7 intrachain disulphide bridge followed by a 23 amino acid chain with a C-terminal prolinamide. The structure of chicken calcitonin has not as yet been determined but it has been shown to have chemical and immunological properties and a potency similar to salmon calcitonin (Neto, Moya and R-Candela, 1973; Cutler, Habener, Dee and Potts, 1974).

Recent studies of the biosynthesis and release of CT from the ultimobranchial gland of the trout (Roos, Bundy, Bailey and Deftos, 1974; Roos, Okano and Deftos, 1974; Roos, Bailey, Okano and Deftos, 1975) and chicken (Nieto, L-Fando and R-Candela, 1975; Moya, Nieto, and R-Candela, 1975) have provided evidence for a higher molecular weight precursor of CT in these species. Such a precursor in the chicken was not however found by Cutler, Habener and Potts (1977) using UB glands from chick embryos, but these authors provided evidence to suggest that two isohormones of chicken calcitonin may exist. In addition Cutler et al discussed the low incorporation of [^{3}H]-leucine into CT, (only about 0.3% of newly synthesized tissue protein is contained in the two CT proteins) and suggested that such a low incorporation rate may be due to the use of embryonic tissue which may well have a large fraction of protein synthesis directed to non-hormonal protein. The alternative is that the UB glands contain a large proportion of cells not associated with CT production.

Whether or not calcium can stimulate directly the biosynthesis of CT in <u>in vitro</u> preparations is contested. Roos, Bundy, Bailey and Deftos (1974) using trout C-cell suspensions and Nieto et al (1975) using cultured UB glands from 8 week old chickens found that increasing the calcium concentration in the medium increased the rate of secretion of CT whilst Feinblatt, Raisz and Kenny (1973) found that any stimulation of CT release by calcium in <u>in vitro</u> 19-20 day chick embryo UB's was dependent upon the oxygen content of the atmosphere surrounding the incubating glands. In particular calcium only stimulated CT release in an atmosphere of 5% CO_2 - 95% air and not in one containing 5% CO_2 - 95% oxygen. Even in the former atmosphere Cutler et al (1977) could find no evidence of an increased release or syntehsis of CT by chicken UB glands,when the external calcium concentration was increased from 1.0 to

3.5 mM. Further work is clearly necessary to resolve this controversy.

Parathyroid Hormone (PTH)

The role of PTH in the control of calcium homeostasis in the chicken has been examined by a number of authors. As in mammals, the parathyroids are involved in the control of plasma ionic calcium; parathyroidectomy results in a fall, and injections of parathyroid extract (including extracts from mammalian species) in a rise, in the plasma calcium level. Candlish and Taylor (1970) found a very rapid response time for exogenous PTH injection in laying hens (less than 10 min).

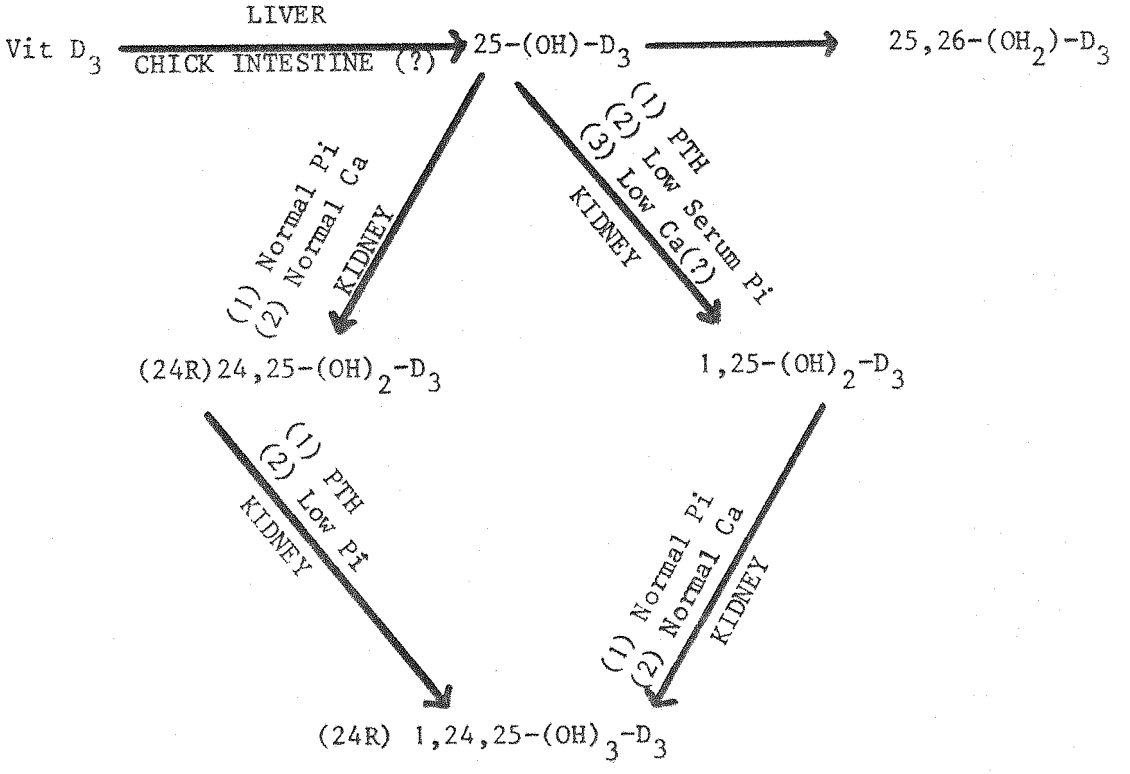
The mode of action of PTH in birds is most probably similar to its actions in mammals, namely an increase in the rates of bone resorption and urinary excretion of phosphate, but one point of dissimilarity is the apparent independence of PTH for vitamin D shown by Hertelendy and Taylor (1960). In mammals the typical hypercalcaemic response to PTH does not occur in rats maintained on a vitamin D deficient diet (Harrison, Harrison and Park, 1958; Marnay and Raoul, 1959).

The involvement of PTH in calcium homeostasis in the chick embryo has been supported by both morphological and experimental evidence (Gaillard, 1959; Narbaitz, 1972 and 1975; Narbaitz and Tellier, 1974; Narbaitz and Gartke, 1975). Embryos after 15-days of incubation respond to exogenous PTH applied to the air-sac membrane by increasing plasma calcium concentration and McFarlane (1965) has shown that glands from 4-5 day embryos explanted into 10-day old chick C-A membranes are capable of secretion, but electron-microscopic evidence suggests that the glands are fully functional by day 17 of incubation (Venzke, 1947). Certainly very young chicks are extremely sensitive to exogenous PTH (Lewis and Taylor, 1972).

Vitamin D.

Metabolism

The central role of vitamin D in calcium metabolism has undergone extensive examination in the past decade. The present state of vitamin D metabolism is well reviewed by De Luca and Schnoes (1976). The metabolism of vitamin D is shown schematically below:



The 25-hydroxylation which takes place in the liver is dependent upon molecular oxygen and is stimulated by reduced pyridine nucleotides (Horsting and De Luca, 1969) and the resulting metabolite, 25-(OH)-D_3, has been shown to be the major circulating form of vit. D bound to an α- or β-globulin or albumin depending upon species, (Edelstein, Lawson and Kodicek, 1972 and 1973; Hay 1975) and to be 2-5 times more active than vit. D_3 itself in the bone and intestine (Blunt and De Luca, 1969; Myrtel, Haussler and Norman, 1970).

Regulation

The most active metabolite is 1,25-$(OH)_2$-D_3, being some 5-10 times more potent than vit. D_3 (Holick, Schnoes and De Luca, 1971; Haussler, Boyce, Littledike and Rasmussen, 1971; Lawson, Frazer, Kodicek, Morris and Williams, 1971) and the regulation of the 1-hydrocylation, (which takes place specifically in the kidney, is thought to be the major site of control although the regulatory factors involved are the subject of controversy and the overall picture is not clear. Regulation by calcium of the enzyme responsible for the 1-hydroxylation has been asserted.

(Boyle, Gray and De Luca, 1971; Ohmdahl, Gray, Boyle, Knutson and De Luca, 1972) and denied (Shain, 1972; Fraser and Kodicek, 1973) and the latter authors suggested that PTH stimulates 1,25-$(OH)_2$-D_3 production directly. CT has been reported to stimulate, (Galante, Colston, MacAulay and Mac Intyre, 1972), inhibit (Rasmussen, Wong, Bickle and Goodman, 1972) or have no effect (Lorenc, Tanaka, De Luca and Jones, 1977) whilst the stimulatory action of PTH has been confirmed (Rasmussen et al, 1972 Garabedian, Holick, De Luca and Boyle, 1972) and been shown to be mediated by an increase in intracellular cAMP (Rasmussen et al 1972). Finally a role for inorganic phosphate has been suggested (Tanaka and De Luca, 1973) in that the 1-hydroxylation activity increases as serum inorganic phosphates falls below 2.6 mM.

One of the alternate metabolites of 25-(OH)-D_3 produced in the same cells as the 1,25$(OH)_2$-D_3 and under the influence of essentially opposite conditions to those favouring 1,25-$(OH)_2$-D_3 production is 24,25-$(OH)_2$-D_3. In birds this metabolite is probably the excretory form of vit. D but in mammals it has been suggested that it may have physiological actions in its own right, for example the 24R configuration (but not the 24S) acts in a manner similar to 25-(OH)-D_3 (Tanaka, De Luca, Ikekawa, Morisaki and Koizumin, 1975) and may also be an important control upon PTH secretion (Bates, Care, Peacock, Mawer and Taylor, 1975), since PTH secretion rate is markedly inhibited when a physiological amount of 24,25-$(OH)_2$-D_3 is added to the perfusate of an isolated parathyroid gland of the goat in situ.

The interrelationship between PTH and vit. D metabolites is of considerable interest. First PTH may stimulate renal conversion of 25 to 1,25-$(OH)_2$-D_3 which then has a direct effect upon the intestine. The 1,25-$(OH)_2$-D_3 is necessary for the action of PTH upon bone and when the plasma calcium level is restored PTH secretion is inhibited both directly via calcium and indirectly via 24,25-$(OH)_2$-D_3.

Mechanism of Action

The mechanism of action of vit. D metabolites has been most studied in the intestine and much argument has centred around the question as to whether or not the action of 1,25-$(OH)_2$-D_3 involves a protein

synthetic event prior to the biological result, namely an increase in calcium transport rate. It has been suggested from studies involving the sub-cellular localisation of 1,25-$(OH)_2$-D_3 and the incorporation of radioactive leucine into newly synthesised protein that the induction of a protein is a primary event (Haussler et al 1971; Kodick, Lawson and Wilson, 1970; Tsai, Wong and Normal, 1972). However Chen, Weber and De Luca (1970) suggested that the 1,25-$(OH)_2$-D_3 was located on the nuclear membrane and not within the chromatin fraction and Tanaka, De Luca, Omdahl and Holick (1971) failed to inhibit the action of 1,25-$(OH)_2$-D_3 upon calcium transport in the intestine by actinomycin D. Furthermore two of these authors (Tanaka and De Luca, 1971) demonstrated that the inhibition by actinomycin D reported by some authors was due to the inhibition of 1,25-$(OH)_2$-D_3 production by the kidney and not its action in the intestine.

Calcium-binding Proteins

One action of vit. D metabolites which is generally accepted is the induction of a calcium-binding protein (CaBP) (Corradino and Wasserman, 1971; MacGregor, Hamilton and Cohn, 1970) although Drescher and De Luca claim that this process is not one of induction, involving the synthesis of CaBP but one of conversion of a non-active to an active CaBP. Other proteins such as alkaline phosphatase and a calcium dependent ATPase may also result from the action of 1,25-$(OH)_2$-D_3 in the intestine, (Haussler, Nagode and Rasmussen, 1970; Holdsworth E.S).

Vitamin D and the Chicken

In the chick intestine system used by Corradino and Wasserman it would appear that the action of 1,25-$(OH)_2$-D_3 (and also 25-(OH)-D_3 and D_3 itself) is indeed an induction process, resulting in the formation of CaBP. Working with isolated chick embryo intestine Corradino et al (1969) found that CaBP was absent until hatching and this result has been confirmed by Moriuchi and De Luca (1974). The latter authors demonstrated the ability of chick embryos to metabolise vit. D_3 to 25-OH, 1,25$(OH)_2$ and 24-25$(OH)_2$-D_3 by at least day 18 of incubation and they suggested that the failure to detect CaBP in the intestine until hatching was due to the very low uptake of 1,25-$(OH)_2$-D_3 by the intestine either due to the absence of the normal mechanism of transport of

1,25-$(OH)_2$-D_3 or due to the lack of its receptors within the intestinal cells. Essentially similar results were obtained by Bishop and Norman (1975) although they detected a sharp rise in 1-hydroxylase activity just before hatching, whilst it was found in the kidney in detectable amounts as early as day 9 of incubation.

Calcium Homeostasis

The regulation of calcium homeostasis is essentially achieved by the control of calcium movement between the following compartments.

(1) The extracellular fluid
(2) The intracellular fluid (and mitochondria)
(3) The bone and bone fluid
(4) The intestinal lumen
(5) The renal tubular fluid

and by the three hormones, PTH, CT and 1,25-$(OH)_2$-D_3.

The early experiments of Copp et al (1961) indicated that in mammals the very precise control of plasma ionic calcium concentration could not be accounted for by the effects of PTH alone and within a few years a new calcium homeostatic hormone was established, namely calcitonin. When Arnaud, Littledike, Tsao (1970) simultaneously measured PTH and CT in young pigs whilst varying their plasma calcium concentration artificially they found that over particular ranges of serum calcium values the concentrations of both PTH and CT in the serum were proportional to serum calcium. Below 3 mM calcium PTH increased with falling calcium and above 2 mM CT increased with increasing calcium and a "cross-over" point occurred at 2.33 mM which is just less than the "normal" circulating total calcium concentration of 2.38 mM.

At about the time of Arnaud's experiments the upsurge in vitamin D investigation began and a third calcium homeostatic hormone, 1,25-$(OH)_2$-D_3 was established, together with the possibility that other vitamin D metabolites may also be involved in the ever increasing complexity of synergisms, inhibitions and other interactions of these hormones and their regulatory factors. The sites of action of these hormones have remained the same, however, although the relative importance of the intestine, bone and kidney, particularly in the minute to minute regulation of plasma calcium concentration is disputed.

Regulation of intestinal calcium absorption

The mucosal uptake of calcium from the intestinal lumen is presumed to be an active, carrier-mediated process in mammalian systems since this influx is inhibited by DNP, iodoacetate and cyanide and requires oxygen (Martin and De Luca, 1969; Papworth and Patrick, 1970; Caspary,1972) but in the chick ileum nitrogen DNP and cyanide have no effect or even stimulate calcium uptake in vitamin D deficient chicks (Adams and Norman, 1970). The influx of calcium in the rat jejunum may also be a passive process and the energy requiring step which may be complicating the general view could be mitochondrial calcium transport. There is no doubt however that the efflux of calcium by the serosal membrane is an active process, occurring as it does against an electrochemical gradient. Calcium transport by the intestine is independent of phosphate (Adams and Norman, 1970; Martin and De Luca 1969), and can readily adapt to changes in dietary calcium intake (Boyle, Gray, Omdahl and De Luca, 1971; Garabedian et al, 1972) via a PTH stimulated production of $1,25\text{-}(OH)_2\text{-}D_3$.

The role of Phosphatases

Calcium sensitive phosphatases (Ca-ATPase) are implicated in intestinal calcium transport at the brush border membrane (Haussler et al, 1970; Holdsworth, 1970; Martin, Melancon and De Luca, 1969; Melancon and De Luca, 1970) where the enzyme is magnesium-dependent, enhanced by potassium and ouabain-insensitive (Melancon and De Luca, 1970) and the activity of this brush border ATP-ase correlates well with $1,25\text{-}(OH)_2\text{-}D_3$ stimulation (Haussler et al, 1970; Holdsworth, 1970; Martin et al, 1969; Melancon and De Luca, 1970). A Ca-ATPase is also required at the serosal membrane where is is essential for active calcium transport: it is enhanced by sodium and inhibited by ethacrynic acid but not by ouabain (Birge, Gilbert and Avioli, 1972; Parkinson, Shami, Hanimyan, Sheepers and Raddle, 1971).

The role of CaBP

The involvement of CaBP in intestinal calcium transport and its regulation by $1,25\text{-}(OH)_2\text{-}D_3$ has been discussed in the section relating to vitamin D. Suffice it to say that the appearance of CaBP correlates

well with,

(1) Intestinal calcium transport
(2) Changes in calcium transport resulting from low calcium (or phosphate) diets (Morrissey and Wasserman, 1971)
(3) The relative absorptive capacities of the various regions of the gastro-intestinal tract (Kallfelz and Wasserman, 1972)
(4) Increased rate of calcium transport in germ-free rats (Reddy, 1972)
(5) Increased rate of calcium transport induced by vitamin D in D-deficient chicks (Ebel, Taylor and Wasserman, 1969).

Adding further support to the involvement of CaBP is the experiment of Corradino and Wasserman (1971b) in which calcium transport in embryonic chick intestine was stimulated by adding CaBP to the medium. It should be mentioned that in this in vitro experiment vit. D(as well as its metabolites) can be used directly to stimulate the intracellular synthesis of CaBP.

There are however two notable non-correlations of CaBP with calcium transport. The first is the persistence of CaBP in response to 1,25-$(OH)_2$-D_3 treatment of rachitic chicks when calcium transport has returned to a baseline level, presumably indicating that the CaBP is no longer functional, or that another factor induced or stimulated by 1,25-$(OH)_2$-D_3 has become limiting (Lawson and Emtage, 1975). The second is the inhibition of vitamin D induced calcium transport by cortisone when injected into rachitic animals without altering the expected increase in CaBP synthesis (Kimberg, Baerg, Gershon and Graudusius, 1971).

Hormonal influences

As well as the effects of 1,25-$(OH)_2$-D_3 upon intestinal CaBP, alkaline phosphatase and Ca-ATPase PTH has been shown to stimulate calcium transport in the intestine (Birge and Gilbert, 1970; Delling, Hehrmann and Montz, 1972; Wills, Wortsman, Pak and Bartter, 1970; Winter, Morava, Simon and Sos, 1970; Olson, De Luca and Potts, 1972) but most authors have found that the effect is not immediate or dependent upon dietary phosphate (Birge and Gilbert, 1970; Olson, De Luca and Potts, 1972; Winter, Morava, Simon and Sos, 1970). Because of the delay in action of PTH some authors have suggested that its action is indirect

and most likely via the stimulation of 1,25-$(OH)_2$-D_3 production, (Tanaka and De Luca, 1973; Garabedian et al 1972), but this is difficult to reconcile with the work of Olson et al (1972) since they were using an in vitro preparation in which an indirect effect of PTH was not possible, but in the authors own admission the dose of PTH required to have an effect was "surprisingly large" (100 U.S.P. units infused acutely).

There is general agreement that CT has little effect upon intestinal calcium transport (Cramer, Parkes and Copp, 1969), and most available evidence indicating an inhibitory role for CT (Hirsch et al 1975; Olson et al, 1972) can be criticised for the non-physiological amounts of CT used to induce any reduction in calcium transport rate.

The results of some experiments reported by Swaminathan, Ker and Care (1974) suggested that CT, at physiological concentrations could reduce intestinal calcium transport probably by inhibiting the 1-hydroxylation of 25-(OH)-D_3. This action of CT was only observed after about two days and it was suggested that the decreased levels of 1,25-$(OH)_2$-D_3 resulting from the CT infusion led to decreased levels of CaBP in the new mucosal cells but did not affect those mucosal cells already functioning at the time of the infusion. This delayed effect could explain the negative results reported by some authors (Cramer et al, 1969). The effect of CT was not due to an indirect action via PTH since essentially similar results were obtained in PTX pigs.

Whilst the experimental results of Swaminathan's experiments are not disputed it would seem from a recent publication (Lorenc et al, 1977) that an inhibitory effect of CT upon 1-hydroxylation of 25-(OH)-D_3 is far from established and thus the interpretation of the mechanism of action of CT in intestinal calcium transport may need to be modified. It might well be that CT does have a long term effect upon the intestine but a role of the action of CT upon the intestine in respect of short term calcium homeostasis seems unlikely.

Glucocorticoids have been shown to inhibit calcium transport without affecting CaBP (Kimberg, Baerg, Gershon and Graudusius, 1971; Krawitt, 1972) or even increasing its concentration (Kimberg et al, 1971), and this may be due to an inhibition of calcium transport at some site other than CaBP induction for instance alkaline phosphatase (Krawitt, 1972).

Regulation of bone resorption

Although the effect of all three calcium homeostatic hormones upon bone resorption is well established and will be discussed below it would be appropriate to establish at the outset our own views upon the role of the bone, particularly the bone apatite crystals, in the minute by minute calcium homeostatic mechanism. It was perhaps Nordin and Peacock (1969) who first re-established the role of the kidney in preference to the bone as the primary calcium regulating organ and other authors have supported the view that the bone may be the "fall-back" organ when sufficient stress is placed upon the calcium homeostatic mechanisms such that the level of plasma calcium can no longer be maintained by alterations in intestinal calcium transport (for instance, when all the available dietary calcium has already been absorbed) and renal tubular reabsorption.

This view has been summarised by Reynolds (1975) and two particular supporting pieces of evidence are worthy of restatement. The first concerns the effect of dichloromethylene diphosphonic acid (Fleisch, Bonjour, Morgan, Reynolds, Schenk, Smith and Russel, 1972; Reynolds, Murphy, Mühlbauer, Morgan and Fleisch, 1973) which blocks the response of bone to the action of 1,25-$(OH)_2$-D_3 but does not result in hypercalcaemia in mice so treated. The second is the ability of 1,25-$(OH)_2$-D_3 to alter plasma calcium concentration in a strain of grey-lethal osteopetrotic mice whose bones do not respond to 1,25-$(OH)_2$-D_3.

There may well be circumstances in which the bone reserves of calcium are essential, (certainly this is the case of medullary bone in the laying chicken) but it would seem that generally bone calcium is conserved and in fact this may well be an important physiological role for CT. One further point which must be made is the concentration of PTH required to act either on the bone or kidney directly or the intestine via 1,25-$(OH)_2$-D_3 production. In an interesting and revealing paper Parsons, Rafferty, Gray, Reit, Zanelli, Keutmann, Tregear, Callaman and Potts (1975) compared reported values for circulating PTH concentration with the lowest dose response of the above three organs and concluded that only the effects upon kidney and gut occur at physiological concentrations of PTH. Interestingly the well established phosphaturia resulting from the action of PTH is much less sensitive, and one could speculate that at high PTH levels, when bone apatite crystals

are resorbed, there occurs a corresponding phosphaturic effect to reduce the unwanted increase in plasma inorganic phosphate concentration which accompanies the desired increase in plasma calcium concentration. Whilst this is highly speculative the situation in the laying hen may serve as an example since the resorption of medullary bone to supply the large calcium requirement of egg shell calcification is known to be accompanied by a renal loss of phosphate, (Common, 1933).

Controversy exists with regard to other aspects of the role of bone in calcium homeostasis. It has been suggested that there is a functional bone membrane separating a bone fluid compartment from the extracellular fluid and that PTH and CT act upon the cells forming this membrane to alter the influx and efflux of calcium between these two compartments. The experimental confirmation of a potassium gradient between these two compartments suggests that such a barrier exists and is capable of maintaining an ion gradient (Canas, Brand, Neuman and Terepka, 1969; Neuman, 1969) whilst the ability of intact bone rudiments to control their external calcium concentration when incubated in vitro and the fact that the entry of calcium into such bone rudiments is independent of phosphate and is a metabolic process inhibited by iodo-acetate adds additional support (Neuman, 1972; Neuman, Mulryan, Neuman and Lane, 1973).

Finally, two processes of bone resorption have been implicated; the first operates via activation of osteoclasts and the second by a process termed osteocytic osteolysis. PTH increases the DNA content within 2 h, (Bingham, Brazell and Owen, 1969; Steinberg and Nichols, 1971) but there is little temporal correlation between the number of osteoclasts and a rise in plasma calcium concentration (Bingham et al 1969; Vitalli, 1968). CT has also been shown to induce morphological changes in osteoclasts within 15 min (Kallio, Garant and Minkin, 1972). Morphological changes have been observed in osteocytes under the influence of PTH within ½-2 h and of CT within 5-8 min (Matthews and Martin, 1971; Matthews, Martin, Collins, Kennedy and Powell, 1972; Jande, 1972; Talmage, Matthews, Martin, Kennedy, Davis and Roycroft, 1975).

Effects of Hormones upon Bone

(1) PTH, the well known action of PTH to induce bone resorption has been shown to require the presence of 1,25-$(OH)_2$-D_3 (Tanaka and De Luca, 1971b) and the involvement of 1,25-$(OH)_2$-D_3 in bone has been convincingly shown to be inhibited by actinomycin D (Garabedian et al, 1974). PTH also stimulates the production of cAMP (Aurbach and Chase, 1970; Chase and Aurbach, 1970; Peck, Carpenter, Messinger and De Bra, 1973) and one of its earliest effects is to increase the influx of calcium into bone cells (Parsons, Neer and Potts Jr., 1971; Parsons and Robinson, 1971; Robertson, Peacock, Atkins and Webster, 1972; Robinson, Rafferty and Parsons, 1972) which is thought to be the cause of the initial transient hypocalcaemic effect of PTH.

The effects of PTH upon osteoclasts and osteocytes have been mentioned above but it should be remembered that the action of PTH may be two fold, firstly to increase bone resorption and secondly to transport calcium from the bone fluid to the E.C.F., (Klein and Raisz, 1971; Meyer and Talmage, 1972; Robertson et al, 1971).

(2) CT. Although the action of CT upon bone has been much studied it would appear that there is as yet no overall agreement as to the mechanism whereby CT inhibits the active bone resorption induced by PTH or whether or not CT is responsible for bone accretion. A number of authors suggest that the inhibitory site of CT is the transference of solubilised calcium from the bone to the ECF (Brand and Raisz, 1972; Minkin, Reynolds and Copp, 1971). Supporting this view is the finding that CT inhibition of bone resorption is not paralleled by a decrease in hydroxyproline (Wener, Gorton and Raisz, 1972). Meyer and Talmage (1972) have suggested that this effect of CT is due to the inhibition of the physiological response to low doses of PTH and that much higher levels of PTH are required to actively cause bone crystal dissolution. Matthews et al (1972) have suggested a possible mechanism of action of CT in the inhibition of calcium transport from the bone which involves the initial movement of phosphate out of osteocytes which results in the formation of amorphous calcium phosphate crystals within the lacunar spaces. It would appear that the efflux of this additional phosphate is sufficient to precipitate calcium phosphate in a medium that is already super-saturated with calcium and

phosphate ions and thus results in an effective removal of ionic calcium to the form in which it is no longer available for transport to the ECF.

The role of osteoclasts in bone resorption is well established (Gaillard, 1957; and Goldhaber 1960) and effects of CT upon these cells have also been observed (Kallio, Garant and Minkin, 1972b). Such changes were morphological in that the ruffled border areas of osteoclasts were flattened and transformed to resemble inactive areas of cytoplasm, and the temporal agreement with the physiological action (i.e. hypocalcaemia) was quite good in that the effects were observable at 15 min and maximal at 1 h. However, as with some of the effects of PTH one must examine carefully the dose of CT required to produce such responses. In Matthew's experiments 100 g rats were injected subcutaneously with 10-25 MRC units of rat or porcine CT and the tibiae of treated and control animals were dissected from freshly killed rats at the various time intervals following the CT injection. Kallio et al used an in vitro cultured 6 day-old mouse calvaria system exposed to a concentration of 166 MRC units/ml of salmon CT for intervals of 1.5 min to 3 h. Apart from being a much higher dose it has been shown that salmon (and other ultimobranchial) CT is much more active in such systems when compared to mammalian CT's (Reynolds, Minkin and Parsons, 1970). Whether or not changes in osteoclasts can be observed at much lower doses of CT is unknown, but it would seem that the experiments of Matthews et al (1972) represent the more physiological approach.

(3) $1,25\text{-}(OH)_2\text{-}D_3$

There is general agreement that $1,25(OH)_2\text{-}D_3$ is the functional form of vitamin D with regard to its bone resorptive action, being up to 100 X more potent than $25\text{-}(OH)_2\text{-}D_3$ whilst vitamin D itself is inactive (Raisz, Trummel, Holick and De Luca 1972; Reynolds, Holick and De Luca, 1973). The action of $1,25\text{-}(OH)_2\text{-}D_3$ on bone is inhibited by CT (Reynolds, 1974) but the involvement of PTH is very much disputed. Whether or not $1,25\text{-}(OH)_2\text{-}D_3$ can act upon bone under conditions where PTH cannot be involved e.g. in parathyroidectomised animals, is still unknown, although such experiments would be difficult since firstly the animals would remain viable for only a short time, secondly removing PTH may well inhibit the synthesis of $1,25\text{-}(OH)_2\text{-}D_3$ and finally it has been shown that bone will have a prolonged response to

both 1,25-$(OH)_2$-D_3 and to PTH after only a brief exposure (Raisz, Trummel, and Simmons, 1972; Rasmussen, 1970). Even in bone culture techniques the response of 1,25-$(OH)_2$-D_3 may still depend upon a prior exposure to PTH. Indeed some authors claim that the action of PTH and 1,25-$(OH)_2$-D_3 are additive and synergistic (Raisz, Trummel and Simmons, 1972).

Calcium Homeostasis and the Kidney

Effects of CT

There is overwhelming evidence to suggest that CT administration to mammals results in the renal excretion of sodium, potassium, calcium, phosphate and water (Singer, Woodhouse, Parkinson & Joplin, 1969; Aldred, Stubbs, Hermann, Zeedyk and Bastian, 1970a; Aldred, Kleszynski and Bastina, 1970b; Haas, Dambacher, Guncaga and Lauffenburger, 1971; Bijvoet, van der Sluys Veer, de Vries, and van Koppen, 1971; Barlet, 1972) and may also result in the renal excretion of citrate (Franklin, Costello, Stacey and Stephens, 1973) and uric acid (Blahos, Osten, Mertl, Kotas, Gregor and Reisenauer, 1975). However, little work has been done to suggest that CT may have similar actions in lower vertebrates and in fact Hayslett, Epstein, Spector, Myer and Murdaugh found no effect of CT upon renal function in the elasmobranch Squalus acanthias, (dogfish shark). In addition it has been shown that CT stimulates a renal adenylate cyclose in the rat (Marx, Fedak and Aurbach, 1972a; Marx, Woodard and Aurbach, 1972b) but not in non-mammalian vertebrates (Dousa, 1974).

The early reports of CT effects upon renal function were criticised by some authors since the effects could have been indirect (e.g. via PTH) and the sources of CT were impure. Such criticism is no longer valid since all the reported effects have been reproduced using synthetic supplies of CT (e.g. Barlet, 1972) and in parathyroidectomised animals (e.g. Aldred et al, 1970a and 1970b) and distinct renal CT receptors have been reported (Marx et al 1972a and 1972b; Sraer and Ardaillou, 1973). The renal effects of CT in relation to calcium and phosphate excretion are thought by some authors to be of particular importance when bone turnover is low (Robinson, Martin, Matthews and MacIntyre, 1967; Cochran, Peacock, Sachs and Nordin, 1970; Haas et al, 1971; Williams, Matthews, Moseley and MacIntyre, 1972) but as already discussed in the section on the regulation of bone resorption, it may

well prove that a renal effect of CT upon the control of plasma calcium and phosphate homeostasis is in preference to any action of CT upon bone resorption.

Effects of PTH

The main renal effects of PTH are well established i.e. increased phosphaturia and decreased calcium clearance, and its mechanism of action involves the binding of the hormone to a distinct renal receptor and the subsequent stimulation of adenyl cylase which results in an increase in the intracellular concentration of cAMP. (Melson, Chase and Aurbach, 1970; Marcus and Arubach, 1971). The most controversial question relates to the doses of PTH used to produce such effects and as already discussed in the section on bone resorption, it may be that the phsophaturic and decreased calcium clearance occur at different dose thresholds, the latter effect being the more sensitive. An interesting aspect of the effect of PTH upon phosphate reabsorption is the parallel inhibition of the reabsorption of sodium, phosphate and bicarbonate in the proximal tubule. (Puschett and Goldberg, 1969; Bijvoet and Froeling, 1973). Although the overall effect upon sodium excretion is minimal, (due to its reabsorption in the distal nephron) the loss of bicarbonate results in an alkaline urine and presumably in plasma acidosis. Although it may be too simple a view it may be that the effect of PTH in reducing plasma pH would seem to result in conditions which would enhance the bone resorptive effects of the hormone. The calcium clearance effect of PTH appears to operate a the site of the distal tubule (Bijvoet et al, 1973).

Effects of Vitamin D

A number of reports suggest that vitamin D, or rather its active metabolites 25-(OH)-D_3 and 1,25-$(OH)_2$-D_3 increase phosphate, calcium and sodium reabsorption in the proximal tubule (Puschett and Goldberg, 1969; Puschett, Fernandez, Boyle, Gray, Omdahl and De Luca, 1972). The latter authors observed that 25 and 1,25-$(OH)_2$-D_3 were equally potent in the kidney although the latter acted more rapidly. Indirect actions of the vitamin D metabolites via PTH have been excluded by using thyroparathyroidectomised animals and the mechanism of action may involve the synthesis of a CaBP (Taylor and Wasserman, 1972; Sands and Kessler, 1971) although this has not been established.

Control of Calcitonin Secretion

Cations

Proportional control of calcitonin secretion rate by serum calcium concentration is well established (Arnaud, Littledike and Tsao, 1970) in mammals as well as lower vertebrates (Ziegler, Delling and Pfeiffer, 1970; Robertson, 1968; Bates, Bruce and Care, 1969). It would seem that in the pig only about one third of the total amount of CT released in response to a hypercalcaemic challenge is newly synthesised since the use of cycloheximide to block protein synthesis results in a 30% decrease in the amount secreted. (Care and Bates, 1972). Similar results were reported by Ziegler et al (1970) for the chicken, who found a 30-50% depression of maximally-stimulated CT-secretion.

A number of authors have reported that the secretory response of C-cells to hypercalcaemia is exaggerated if the cells have been previously exposed to hypocalcaemia and this effect may be of physiological significance (West, O'Riordan, Copp, Bates and Care, 1973; Heynen and Franchimont, 1974). This exaggerated response can also be obtained if animals are fed a normal calcium diet after a period of calcium deficiency.

The effect of hypercalcaemia on CT secretion in the Japanese quail, hen and goose *in vivo* is undisputed (Boelkins and Kenny, 1973; Ziegler et al, 1970; Bates et al 1969, respectively) but the ability of chicken UB glands to respond to a calcium challenge *in vitro* is contested. Feinblatt et al (1973) found that the response was dependent upon the oxygen content of the atmosphere surrounding the UB cultures of embryonic glands and Nieto et al (1975) found a 2-8 fold increase in the rate of secretion of CT in 8 week chick UB glands. However, Cutler et al (1977) found no effect of calcium upon the secretion of CT by the UB glands of newly hatched chicks.

The mechanism by which hypercalcaemia raises the secretion rate of CT has inevitably involved the ubiquitous cAMP. It has been reported that when the intracellular levels of cAMP is increased by treating mammalian C-cells with dibutyryl cAMP or inhibitors of phosphodiesterase, the enzyme responsible for the breakdown of cAMP, the rate of CT secretion is increased (Kaltenbach, Graber, Niswender and Nalbandov, 1968) and similar results have been obtained by Ziegler et al (1970) in the hen. The role of cAMP in addition to stimulating the release of

CT may also involve stimulation of its synthesis (Bell and Queener, 1974).

In addition to calcium, both magnesium and strontium have been shown to stimulate CT secretion, (Care et al 1971a and Pento et al, 1974; Bates et al, 1974). In both cases the concentration required is well beyond the normal physiological limit of these ions although Corradino et al (1971c) have suggested that the effect of strontium may be of some importance in cases of strontium rickets.

Hormonal Control

In 1969 Gray and Munson demonstrated the hypercalcaemic effect of oral calcium in the thyroidectomised (TX) rat, whereas no significant changes in plasma calcium were detected in thyroid-intact rats. Lederer, Stein and Arnould (1969) reported the hypercalcaemic effect of high calcium diets in TX animals. Munson et al (1971) infused calcium chloride into the jejunum of rats in doses which were too small to increase the peripheral plasma calcium concentration but found a significant increase in CT secretion. Their initial interpretation was that the C-cells were very sensitive to small (undetectable) changes in plasma calcium concentration but this view has now been modified due to the establishment of a gastro-entero-thyroid C-cell system involving the direct stimulation of CT secretion by some of the gastro-intestinal (GI) hormones released in response to the presence of food in the GI tract.

Pancreozymin or its C-terminal tetrapeptide has been shown to stimulate CT secretion. This C-terminal tetrapeptide is common to gastrin, pentagastrin, tetragastrin and caerulan and all these hormones stimulate CT secretion (Care et al, 1971a and 1971b; Cooper et al, 1972). The most convincing experiments for a role of GI hormones in stimulating CT secretion and thereby preventing post-prandial hypercalcaemia have been performed by Swaminathan et al (1973). In these experiments endogenous secretion of enteroglucagon, pancreozymin-cholecystokinin, and gastrin was induced by glucose, fat (as cream) and glycine respectively and in each case there was an increase in CT secretion rate. In addition the C-cell response to increased calcium concentration was greater than normal if the thyroid glands were previously exposed to one of the above GI hormones and it may be that

this sensitising effect is of physiological importance in post-prandial calcium homeostasis.

The existence of a gastro-entero-thyroid C-cell system has not been confirmed in avian species. However, Care and Bates (1973) reported that caerulein, at a dose sufficient to stimulate CT secretion in the pig, was without effect in the goose. It should be noted that one might only expect such a system to operate in meal eating as compared to "nibbling" animals, in which case one might not expect such a system to be of importance in the chicken, since the presence of a crop ensures the almost constant passage of food through the GI tract. In this respect it is interesting to note that there is little effect of feeding upon CT secretion in a ruminant, the sheep, in which there is also a continuous flow of food from the fore stomachs to the abomasum (Phillipo, Lawrence, Bruce and Donaldson, 1972).

In addition to the G-I hormones, adrenaline has been shown to stimulate CT secretion by a β-adrenergic pathway in the pig, sheep and dog (Care et al, 1971b; Bates et al, 1970; Avioli, Scott, Shieber and Kipnis, 1971). However, the physiological significance of this effect of adrenaline is unclear. Other hormones which have been tested for CT-secretion activity include PTH, 1,25-(OH)-D_3 and 24,25-$(OH)_2$-D_3, but all were without effect (Care et al, 1975).

Neural Control

Hodges and Gould (1969) demonstrated that electrical stimulation of the left vagus in young anaesthetised cockerels was associated with a significant fall in plasma calcium concentration which they suggested might be indicative of an increased rate of CT secretion. More direct evidence came from the measurement of CT levels in the plasma of vagal-stimulated cockerels (Gould and Hodges, 1970). Care and Bates (1973) performed similar experiments in the goose and found that vagal stimulation increased the rate of CT secretion, despite a small fall in plasma calcium concentration. It would therefore appear that a neural pathway for CT secretion may be of importance, at least in avian species, and this possibility is supported by histological evidence. However, there is no experimental evidence in which neural stimulation of CT secretion has been shown to occur naturally and the physiological conditions which might induce neural stimulation remain unknown.

Calcium homeostasis in the Chick Embryo

Hormonal control of plasma calcium homeostasis in the chick embryo has been little studied but some control has been inferred from the relatively constant plasma calcium concentration (Narbaitz, 1975; Stewart and Terepka, 1969). The results of Taylor (1963) suggested that there was an increase in plasma calcium concentration with incubation time from a very low value (< 1.5 mM) at day 14 to "normal" values (about 2.5 mM) at hatching. The low calcium concentrations were associated with high magnesium values and vice versa. It would now appear that these results are invalid and due to the methodology adopted by Taylor for calcium and magnesium determination, namely an EDTA titration. The reason why this EDTA titration method, (which gives accurate values in young chick , hen and mammalian plasma), fails to give accurate values for plasma calcium when embryonic chick plasma is used, has not yet been determined.

Assuming then that plasma calcium remains relatively constant during the last week of incubation then it would appear that some controlling mechanisms must be operating in the chick embryo to maintain plasma calcium homeostasis since this is the period when large amounts of calcium are transferred from the egg-shell to the rapidly calcifying skeleton. During the last seven days of incubation there is a net transfer of over 125 mg of calcium from the shell and yolk-sac, to the embryonic circulation and then to the skeleton (Johnston and Comar, 1955).

It has been suggested that the control of plasma calcium may be exerted, at least in part, by a physio-chemical mechanism involving the deposition of excess calcium into the yolk-sac (Johnson and Comar, 1955) whilst the active transport of calcium by the chorio-allantoic (C-A) membrane proceeds at a maximal rate (Terepka, Coleman, Garrison and Spataro, 1971). No evidence is available concerning hormonal influences upon calcium transport via the C-A membrane but it has been assumed that the process must require some hormonal activation and the failure of PTH and CT to alter the calcium transport rate in vitro may be due to the fact that the membrane has been maximally stimulated prior to its removal for in vitro studies (Terepka et al, 1971).

The only direct evidence for hormonal influence upon plasma calcium concentration in the chick embryo has come from Narbaitz (1975).

He demonstrated the ability of PTH to increase plasma calcium concentration when applied to the air-sac membrane of 15 day old embryos. The possible effects of CT and vitamin D metabolites has not been investigated, although it has been known for a number of years that vitamin D is required for successful embryonic development (Hart et al, 1925). In addition it has been demonstrated that the chick embryonic kidney is capable of metabolising 25 to 1,25-$(OH)_2$-D_3 (Moriuchi and De Luca, 1974; Bishop and Norman, 1975). The latter authors found detectable levels of 1,25-$(OH)_2$-D_3 in day 17 embryos *in vivo* although the capacity for 1-hydroxylation was present at day 9 *in vitro*.

The possible involvement of CT in the chick embryo was first suggested by Lewis and Taylor (1972) and Taylor, Balderstone and Lewis (1975). CT was detectable by bioassay in chick embryos from day 17 of incubation and until hatching, reaching very high levels during day 20. These results have been confirmed by Cutler, Habener, Dee and Potts (1974) using a radioimmunoassay and the latter authors also investigated the total amount of CT in the UB glands of embryos and found an enormous increase towards the end of incubation which continued after hatching.

Calcium Homeostasis in the Chicken

All three hormones associated with calcium homeostasis in mammals are present in chickens although the CT secreting cells are found in the distinct ultimobranchial glands. In respect of 1,25-$(OH)_2$-D_3 and PTH it would be generally true to say that the mode of action of these hormones and their regulatory factors in the chicken are similar to those in mammals. The only notable point of difference relates to the time course of action of PTH. Candlish and Taylor (1970) demonstrated the much more rapid action of PTH in the chicken, since hypercalcaemia was detectable within 8 mins of PTH administration although the effect was shorter than in mammals.

The UB glands hypertrophy in the laying hen and also when birds are fed a high calcium diet (Urist, 1967; Cipera, Chan and Bélanger, 1970). Perfusion of isolated chicken UB glands with hypercalcaemic blood resulted in an increase in CT secretion rate (Ziegler et al, 1970; Bates et al, 1969) and infusion of calcium into anaesthetised young turkeys also resulted in an increase in circulating CT. Thus it would seem that the UB gland is functional in calcium homeostasis in the

chicken. However, the ability of CT to reduce plasma calcium concentration below normal levels in the chicken is very much disputed. A number of authors have failed to obtain a hypocalcaemic effect with either mammalian CT or chicken UB extract (Urist, 1967; Candlish and Taylor, 1970; Gonnerman et al, 1972). The latter authors also failed to achieve a hypocalcaemic response in PTX birds, nor could they modify the effect of exogenous PTH by simultaneous administration of CT or UB extract.

Kraintz and Intscher (1969) reported a hypocalcaemic effect of CT in PTX but not in intact fowl but their experiments can be criticised for the following reasons:

(1) the limited number of animals used

(2) the absence of any statistical analysis of data

(3) the use of anaesthetised animals maintained upon an artificial respirator.

A hypocalcaemic action of UB extract in intact 7 day old cockerels and 12 week chickens (but not laying hens) was reported by Lloyd, Peterson and Collins (1970). However, a detailed examination of their results leads to the conclusion that this effect of UB extract is far less convincing than the authors themselves conclude. In the case of their 7 day old cockerel results the control plasma calcium values ranged from 9.8 to 11.1 mg% whilst the experimental values ranged from 10.4 to 10.5 mg% at all time intervals (15, 30, 60 and 120 min) except for 90 min post-injection when the control and experimental groups were 9.8 and 9.6 mg% respectively. Similarly, in the case of the 12 week old chickens examined at the same time intervals as above, UB extract produced a hypocalcaemic response at 90 mins but this effect was only 0.1 mg% less than the mean zero-time concentration whereas the control value at the same time had risen by 0.9 mg%.

Whether or not CT is able to reduce plasma calcium from normal to low levels is clearly debatable but there are some interesting experiments reported in the literature which suggest that CT may function in a restrictive manner in the chicken. The most convincing experiment was performed by Copp et al (1970) in which calcium was infused into a young anaesthetised turkey and midway through this infusion the UB glands were surgically removed. The result of the removal of the UB glands was a rapid increase in circulating plasma calcium concentration

even compared to the high plateau calcium concentration obtained initially but in the presence of UB glands. Supporting a restrictive role for CT are the experiments of Brown et al who removed UB glands from young chicks and found that whilst this had little effect upon base line plasma calcium the ability of these chicks to control a PTH induced hypercalcaemia was impaired.

Apart from general considerations of the role of CT, PTH and $1,25\text{-}(OH)_2\text{-}D_3$ in the normal calcium homeostasis of the fowl particular attention has been directed to the possible role of these hormones in egg-shell calcification. Because the skeletons of chickens are light in weight in order to facilitate flight the proportion of calcium required for the calcification of a single egg-shell (approx. 2 g) is very high compared to the total available calcium in the chicken (approx. 20 g) and must place an enormous stress upon calcium homeostasis. Certain adaptations have necessarily taken place to relieve this stress of which two are worthy of further discussion in this review. The first is medullary bone which is formed under the influence of both oestrogen and androgens and which appears to act as a labile reservoir of calcium. The second is an adaptation to increase the efficiency of absorption of calcium in the intestine such that the dietary calcium may supply up to 2 g calcium per day (Hurwitz and Bar, 1969).

Whilst the factors responsible for the formation of medullary bone are well understood (for a review see Simkiss, 1967; Taylor, 1970), the mechanisms controlling the resorption of medullary bone has been contested. The first theory was put forward by Riddle et al (1945) and suggested that resorption is brought about by a reduction in the level of oestrogen circulating in the blood after ovulation. However this theory was based upon work with the pigeon which lays a clutch of only two eggs on successive days in a single year, (a hen can lay up to 250 eggs per year!) and in this species it is quite likely that there is a fall in oestrogen levels after the second ovulation. This need not be the case in the chicken and direct measurements of oestrogen at different stages of the ovulatory cycle have not supported a theory of oscillating oestrogen levels coinciding with periods of egg-shell calcification, (Senior and Cunningham, 1974; Peterson and Common, 1972).

An alternative theory has been put forward by Taylor to account for medullary bone resorption (Taylor, 1965 and 1970). This theory

suggests that a fall in plasma ionic calcium due to the onset of egg-shell calcification leads to the stimulation of PTH release, the levels of which controls the rate of medullary bone resorption. Clearly the best experimental proof of this theory will require the direct measurement of circulating PTH at different times of the ovulatory cycle but this experiment has not yet been carried out.

There is some experimental evidence which would seem to suggest that a role for PTH in medullary bone resorption is unlikely. Perhaps the major discrepancy arose from an experiment by Taylor and Moore (1954) in which hens were fed calcium deficient diets but the amount of medullary bone was maintained, (although the % ash was much reduced), at the expense of cortical bone. This seemingly contradicts an essential feature of the PTH theory namely that medullary bone should be more sensitive to PTH compared with cortical bone and thus one should expect its almost complete resorption under conditions of high circulating PTH levels. A possible explanation has been put forward by Taylor (1970) which suggests that under a normal (or high) dietary calcium intake circulating PTH levels are low and small increases in PTH due to the onset of egg-shell calcification have a substantial (probably approaching maximal) effect upon medullary bone but little effect upon cortical bone. However, under a low dietary calcium intake circulatory levels of PTH will already be high and further increases due to egg-shell calcification may greatly increase the response of cortical bone whilst any increase in the response of medullary bone will be slight since the maximal rate of resorption has already been reached. In other words, the PTH theory requires that medullary and cortical bone have different dose thresholds for PTH, the latter being less sensitive than the former but under conditions of low dietary calcium intake cortical bone is resorbed in order to maintain medullary bone.

Since PTH has been implicated it may be that CT also functions in the control of medullary bone resorption. One could speculate that on cessation of egg-shell calcification a rise in plasma ionic calcium would stimulate CT secretion and the increase in circulating CT may then inhibit PTH induced medullary bone resorption. Once again the most obvious test of this possibility would be the direct measurement of circulating CT during the ovulatory cycle. Such an experiment has been done by Dacke, Boelkins, Smith and Kenny (1972) using mature, laying,

Japanese Quail. The results indicated high plasma CT concentrations early in the ovulatory cycle, when no egg-shell calcification was taking place but lower levels later in the cycle. Thus a role for CT in calcium homeostasis in the laying bird has rec ived some experimental confirmation. However, Speers, Perey and Brown (1970) had previously shown that chicks UBX at 1-2 days of age and reared until egg-laying still produced relatively normal eggs. There was a slight tendency for the eggs of UBX chickens to be smaller and to have thinner shells compared to sham operated controls, but these effects could be explained by the reduced food intake of the UBX birds. A role of CT in calcium homeostasis during the ovulatory cycle has therefore yet to be convincingly established.

Possible changes in vitamin D metabolism during egg-laying have also been investigated (Kenny, 1976). It would appear that an increase in the activity of renal 25-(OH)-D_3 1-hydroxylase follows ovulation in the Japanese quail and this increase is maintained for at least 24 h and is present even during the first 1-6 h when no egg-shell calcification is taking place. If oviposition is not followed by a further ovulation then the 1-hydroxylase falls rapidly. Similar results have been obtained in the chicken. These observations are interesting not just in respect of calcium homeostasis during the ovulatory cycle but also because they represent the first evidence of natural modulations in the 1-hydroxylase activity which are almost certainly induced hormonally, most probably by oestrogen. However, Sedrani and Taylor (1977) have disputed Kenny's findings and have suggested that the regulatory factor involved in controlling 1-hydroxylase activity is PTH and not oestrogen.

CHAPTER 2

METHODS AND MATERIALS

2.1 Incubation of fertile eggs

Fertile eggs (obtained from Sterling Poultry, Upper Clatford, Nr. Andover, Hants, England) of a heavy breed of domestic fowl (ROSS I) were incubated in a forced-draught incubator ("Westernette" and "Curfew" Messrs Western Incubators Ltd., East Hanningfield, Essex, England), maintained at a temperature of 37.8°C and fitted with an automatic humidifier (which maintains the humidity at 60%) and an hourly turning device. After seven days of incubation eggs were candled and infertiles removed. Eggs used experimentally were kept in a Western "Curfew" (Messrs. Western Incubators Ltd., East Hanningfield, Essex, England) observation incubator maintained at the same temperature and humidity as above. Newly hatched and older chicks were also kept in an observation incubator. Incubation was always started at 5 pm on day 0 to ensure that the three physiological stages identifiable on day 20 occurred during the normal working day. The majority of chicks hatched overnight on the 20/21st day or during the morning of day 21. The measurement of the third toe length was used as a guide to the development of the embryos (Hamburger & Hamilton, 1951).

2.2 Radiochemicals and Isotope counting methods

All radiochemicals were obtained from the Radiochemical Centre, Amersham, England. The following isotopes were used and details of the method of counting given;

Calcium - 45 (as chloride)	Liquid Scintillation
Sulphur - 35 (as sulphate)	" "
Carbon - 14 (^{14}C-mannitol)	" "
Phosphorus - 32 (as phosphate)	" "
Iodine - 125 (as NaI)	Gamma Counter

Liquid scintillation counting was done using a Philipps Liquid Scintillation Analyser Model No. PW 4510/01 or a Beckman(R) Scintillation Counter, Model 1650 and Gamma counting involved the use of a Beckman(R) Biogamma(R) Counter.

The liquid scintillation fluid used throughout was a "home-made" cocktail designed for aqueous samples and consisting of the following:

Xylene	666 ml
Synperonic NXP	333 ml
2,5,-Diphenyloxazole (PPO)	4g
1,4-Di- 2-(4-methyl-5-phenyloxazolyl) -benzene (Di-methyl POPOP)	0.3 g

(Synperonic NXP is a non-ionic detergent available from Cargo Fleet Chemical Co., Eagles Cliffe, Stockton, England.)

The following table summarises further details of the isotopes used:

Isotope	Emission	Energy (MEV)	$t_{\frac{1}{2}}$
^{45}Ca	β^-	0.256	163 d
^{35}S	β^-	0.167	86.7 d
^{14}C	β^-	0.156	5770 y
^{32}P	β^-	1.7	14.5 d
^{125}I	γ	0.035	60 d

The efficiency of counting of ^{45}Ca and ^{32}P by liquid scintillation was always greater than 96% and no correction was made in order to present results as dpm rather than cpm. However, suitable controls of known cpm were always mixed with the type of sample to be counted, i.e. plasma, acid digests etc., to ensure that effects of quenching would be detected, but no corrections had to be made with the volume and type of samples used in all the experiments reported in this thesis. Both ^{14}C and ^{35}S counts were corrected automatically by computer from previously programmed quench and efficiency curves to give results in terms of dpm. The efficiency of counting of ^{125}I was greater than 85% and no corrections were made to convert cpm to dpm. The ^{125}I used was specifically for protein iodination (Code IMS.30) and consisted of sodium iodide in dilute NaOH solution pH 8-11.

In one experiment ^{45}Ca and ^{32}P were counted in the same sample using a Beckman scintillation counter with suitable channels for the separation of these two isotopes. The large difference in the energy of emission facilitated virtually complete separation of these two isotopes.

2.3 Determination of Calcium and Magnesium

Both of these elements were determined using an Atomic Absorption Spectrophotometer (AAS) (Pye-Unicam SP90A Series 2 A.A.S, Pye-Unicam Ltd. Cambridge, England) with specific calcium and magnesium lamps. Samples of 50 or 100 µl were diluted with 50 volumes of a solution of lanthanum chloride containing 10 m-mole La Cl_3 and 50 m-mole HCl/1 (Pybus, Feldman and Bowers, 1970). Standards were prepared from An lar Calcium Carbonate (British Drug Houses, Poole, England) and contained the same concentration of $LaCl_3$. The latter was added to samples (particularly plasma or serum) to suppress interference by phosphate. Where possible glassware was avoided and all samples for analysis were kept in stoppered 5 ml plastic sample tubes and standard calcium and magnesium solution were stored in plastic containers.

2.4 Determination of Sodium and Potassium

Sodium and potassium were determined by flame photometry using an EEL Integrating flame photometer, model 227 (Evans Electroselenium Ltd Halstead, Essex, England). Stock and working solutions were as follows:

Stock Solutions

Li_2CO_2	150 mM Li^+ (5.541 g of 99.8% pure Li_2CO_3 in min. vol 5 M HCl then diluted to 1 l with deionised water)
NaCl	200 mM Na^+ (11.688 g A.R. NaCl/1)
KCl	100 mM K^+ (7.456 g A.R. KCl/1)

Working Solutions

High Standard: 2 mM Na^+, 1 mM K^+ 15 mM Li^+

i.e. 100 ml Li_2CO_3, 10 ml NaCl, 10 ml KCl } to 1 l

Low Standard: 15 mM Li^+

i.e. 100 ml Li_2CO_3/1

Sample Preparation

Samples were diluted 1:100 and included 0.5 ml of the stock Li_2CO_3

solution i.e. for 50 µl of plasma:-

50 µl sample
0.5 ml Li_2CO_3
4.45 ml H_2O
5 ml total volume.

2.5 Determination of inorganic phosphate

Two methods were employed for the determination of inorganic phosphate,

(1) Chen, Toribara and Warner (1956) - a colorimetric method, reading the absorption at 920 nm of an ascorbic acid reduced phosphomolybdate complex. The determination was carried out on protein-free filtrates of plasma which had been precipitated with 10% trichloracetic acid (cold)

(2) Autoanalysis - using a Technicon(R) Autoanalyser(R) (Technicon Inc., Tarrytown, N.Y, USA.) The method is based on the formation of phosphomolybdic acid which is reduced by stannous chloride-hydrazine (Hurst, 1967). The method was linear over the range 0-3 mM phosphate using a KH_2PO_4 standard solution.

2.6 Determination of plasma and ECF volume

(1) Plasma Volume: was determined by a dye dilution method using Pontamine Sky Blue, (PSB), bound to Bovine Serum Albumin (BSA). The following solutions were used:

Pontamine Sky Blue: 5 g/l in 9 g/l NaCl and containing 50 g/l BSA (filtered before use)

Blank and Standard Curve Diluent: 50 g/l BSA in 9 g/l NaCl.

Method

0.1 ml PSB was injected i.v. into chick embryos and a blood sample withdrawn after exactly 5 min. A small volume of this blood was used for the determination of the haemotocrit and the remainder centrifuged and 3 × 0.1 ml samples of plasma were added to 3 ml distilled water. Standards were prepared using 0.1 ml of the same PSB solution added to various volumes

(up to 3.5 ml) of the BSA/NaCl diluent. 0.1 ml of each standard was then added to 3.0 ml distilled H_2O and the absorption at 600 nm read against a blank containing 3 ml distilled H_2O and 0.1 ml of the diluent.

The plasma volume was read directly from a standard curve but if the blood volume was required it was calculated using the figure for the haematocrit of the same sample from the following expression:

$$\text{Blood Volume} = \frac{\text{Plasma Volume}}{1-\text{Hameatocrit}}$$

Care was taken in the preparation of plasma from the blood samples to ensure that no haemolysis occurred i.e. the minimum centrifugal force was employed to separate plasma from blood cells.

(2) ECF Volume: was determined by the isotope dilution of either $^{35}SO_4$ or ^{14}C-mannitol. The advantages and disadvantages of using two isotopes are discussed in detail in Experiment 6.

2.7 Storage of Samples

All plasma samples awaiting CT determination were stored in small plastic sample tubes under liquid nitrogen. Samples for phosphate determination were stored at $-20^{\circ}C$ and diluted samples for Ca, Mg, Na and K were stored at $4^{\circ}C$.

2.8 Injection of fertile eggs

(1) Albumen Injection

Eggs in which the albumen was to be injected were first candled and a spot marked on the "sharp" end of the egg which avoided any major blood vessel. A 100 µl syringe with a long needle marked at 1.5 cm was used for the injection and a dental drill was used to drill a small hole to the level of the shell membrane. Solutions to be injected were autoclaved and as sterile a procedure as possible was followed. The needle was inserted to the 1.5 cm mark and the contents injected slowly. After injection the hole in the shell was covered with a small drop of molten wax and a small (1 cm^2) piece of radioactive warning tape.

(2) Intravenous Injection

Eggs to be injected were first candled to locate a fairly large, straight vein embedded in the chorioallantoic membrane. The area was marked and a small piece of shell (1 × 1.5 cm) was removed by first cutting through the shell with a glass-cutting blade (or other suitable serated edge) and the shell was carefully separated from the underlying membranes with a small spatula. A drop of sterile light mineral oil was then applied to the exposed shell membrane to render it transparent. The egg was then viewed under a binocular dissecting microscope and the direction of blood flow determined. A 2.5 cm × 27 gauge dental needle, connected to a 25 gauge needle and 1 ml syringe via a 10 cm piece of PVC cannula tubing of a suitable size, was used for the injection. It was essential to inject in the direction of blood flow to prevent haemorrhage on removal of the needle. The angle of insertion was as acute as possible, using the edge of the cut shell as a support and keeping the bevel of the needle uppermost. The solution was injected slowly and the needle then withdrawn rapidly. If any excessive bleeding occurred then such eggs were rejected. A small volume of blood (estimated to be no greater than 20 µl) often covered the site of injection and was tolerated.

(3) Air-sac injection

A small hole was drilled in the shell surrounding the air-sac and a 25 gauge needle used for the injection of the particular solution which was dropped slowly onto the air-sac membrane. The eggs were returned to the incubator with the hole sealed with a drop of molten wax and were supported with the air-sac uppermost.

2.9 Collection of blood samples

(1) Embryos

(a) Days 14-16 of incubation: The shell was carefully removed from the egg, particular care being taken to avoid excessive bleeding from the chorio-allantoic vessels. The major umbilical artery is clearly visible up to day 16 and a 26 gauge needle was inserted at the point of the first bifurcation of the artery, against the blood flow. Blood (up to 0.8 ml) was collected into heparinised 1 ml syringes.

(b) Days 17-20 of incubation: The shell was removed and the membranes surrounding the embryo were cut at a point where little

vascularisation occurred to prevent excessive bleeding. The embryo was carefully removed and the thorax exposed by dissection and blood collected by cardiac puncture using heparinised 25 g needles and 1 ml syringes. It was particularly useful to remove the pericardium at the point of insertion of the needle, i.e. the tip of the ventricle, to prevent blockage of the needle by this membrane. All pulmonary respiring chicks were lightly anaesthetised with diethyl ether.

(c) Newly hatched and older chicks: Blood was collected by direct cardiac puncture of anaesthetised chicks without exposing the heart.

(2) Chickens

Blood was collected into 2 ml heparinised syringes by venipuncture of a wing vein using 23 gauge needles. It was particularly useful to remove the feathers covering the vein to be used and to clean this area with a solution of ethanol and distilled water (7:3 vol/vol) which served both to clarify the skin covering the vein and to sterilise the area. The greatest success, with least post-bleeding haemorrhage, was achieved by introducing the needle at as acute an angle as possible with the bevel of the needle downwards. On removal of the needle pressure was applied to the area in order to prevent haemorrhage.

(3) Rabbits

Up to 10 ml of blood was collected from a cut ear vein of a rabbit after having previously warmed the ear under a desk lamp (60 w bulb). Rabbits were handled as gently as possible and on completion of the blood collection a small piece of cotton wool was affixed to the cut area with a paper clip for up to 10 mins or until such time as all bleeding had stopped. The blood was allowed to drip directly into heparinised plastic centrifuge tubes. To facilitate blood collection it was useful to shave an area of about 1 cm^2 above the vein to be used.

2.10 Treatment of Blood Samples

Heparin (Available from The Boots Co. Ltd., Nottingham, England) was used routinely as an anticoagulant at a final concentration of approximately 400 units/ml blood. If small samples of blood (up to 1 ml) were collected then LP3 (Luckhams Ltd., Burgess Hill, Sussex, England)

plastic tubes were used and the samples were kept on ice prior to centrifugation to separate plasma and blood cells. Larger volumes of blood were collected in conical plastic centrifuge tubes and were also kept on ice before centrifugation.

2.11 Ashing of materials

(1) Dry : Embryos and Yolk-sacs.

Embryos and their associated yolk-sace were removed from fertile eggs and separated at the umbilical stalk. Each part was then carefully washed with deionised water to remove any traces of albumen and then placed in a preweighed porcelain crucible and dried in an oven at 110°C for at least 2 days. Prior to ashing overnight in a muffle furnace at 600°C samples were charred in a bunsen flame to prevent "spitting". After ashing crucibles were allowed to cool in a desiccator and then reweighed in order to determine the ash weights. The contents were then dissolved in 3 × 2.5 ml 5.5 M HCl, transferred quantitatively to a 10 ml volumetric flask and volume made up to 10 ml with deionised water. Samples of these solutions were then taken for total calcium and in some cases ^{45}Ca determination.

(2) Wet : diet samples.

Preweighed samples of diets (10 g) were refluxed in concentrated nitric acid for 6 h, allowed to cool and the contents together with washings transferred quantitatively to a 100 ml volumetric flask and suitable samples removed for calcium and phosphate determinations.

2.12 Chicken Calcitonin Radioimmuno-assay

The RIA used was based upon the cross-reactivity of salmon and chicken calcitonins first shown by Cutler, Habener, Dee and Potts (1974).

(1) Antibody Production

A minimum of 10 µg of synthetic salmon calcitonin (sSCT; Lot K668-280, activity 4790 u/mg; a gift from Armour Pharmaceutical Co. Kankakee, Ill, USA) contained in 0.33 ml distilled water was mixed with 0.66 ml Freunds Complete Adjuvant (Difco Laboratories, Detroit, Michigan USA) and injected intra-dermally at multiple sites into each of three cross-

bred rabbits. The method of injection was chosen because of the small amount of sSCT available (Vaitukaitis, Robbins, Nieschlag and Ross; 1971). To assay the titre of antibodies 5 ml of blood was collected from an ear vein and the heparinised plasma was diluted 1:10 with 0.4 M tris/HCl buffer, pH 7.4 containing 1.0 g/l BSA and 0.2 g/l thiomersal (an anti-microbial agent). Aliquots of 1 ml diluted plasma were stored under liquid nitrogen in glass vials.

(2) Iodination of sSCT

Radioiodination of sSCT was achieved by the method of Hunter and Greenwood (1962) with minor modifications. The following solutions were required:

(1) 0.5 M $NaH_2PO_4.2H_2O$ pH 7.4
(2) 0.05 M $NaH_2PO_4.2H_2O$ pH 7.4
(3) (2) as above + 2 g/l B.S.A. (elution buffer)
(4) 5 g Sephadex(R) G-25 medium (Pharmacia Fine Chemicals, Uppsala, Sweden) soaked in the elution buffer at 4°C and then packed into a 1 × 30 cm glass column and maintained at 4°C.
(5) Choramine T, 2 ml of a 5 mg/ml solution in (2)
(6) Sodium Metabisulphite, 2 ml of a 24 mg/ml solution in (2)
(7) Potassium Iodide, 2 ml of a 10 mg/ml solution in (2)
(8) sSCT, 5-10 µg in 10 µl saline (9 g/l NaCl)
(9) Iodine-125. IMS 30 (Radiochemical Centre, Amersham, England) - 2 m Ci ^{125}I as sodium iodide in dilute NaOH solution pH 8-11.

Method

The entire iodination procedure was carried out in a cold room at 4°C, to which all solutions were equilibrated before commencement.

The following were added to the vial containing the ^{125}I IN THE CORRECT ORDER

(a) 10 µl of (1) above
(b) 10 µl (5-10 µg) sSCT
(c) 10 µl chloramine T
(d) (WITHIN 15 sec of c) 10 µl Na Metabisulphite
(e) 0.2 ml KI

The contents of the vial were then placed onto the surface of a Sephadex G-25 column with a long tipped pasteur pipette. A further 0.2 ml KI was used to rinse out the iodination vial and was also added to the column, which was then eluted with the albumen/phosphate buffer and 5 drop fractions were collected into LP3 polystyrene tubes (Luckhams Ltd., Burgess Hill, Sussex, England) until all the ^{125}I had been eluted from the column. The tubes were then counted for 1 sec in a Gamma Counter. The fractions with the highest activity, i.e. the first peak which elutes from the column were pooled and then divided into 1 ml aliquots in glass vials and stored under liquid nitrogen. The elution profile from a typical iodination is shown in fig. 2.1.

A small sample of the iodinated sSCT was counted in a Gamma Counter and then the protein was precipitated with 10% Trichloracetic acid (cold), the sample centrifuged and the supernatant and protein precipitate counted separately. The precipitability of the ^{125}I in a good iodination exceeded 95%.

Specific activities of 150-300 μCi/μg were achieved and the ^{125}I-CT was usable for up to 6 weeks (see Fig. 2.1). Electrophoresis on cellulose-acetate strips was used to estimate damaged protein and between 90 and 94% of the total radioactivity remained in a peak at the origin. Electrophoresis of iodinated protein after 6 weeks of storage under liquid nitrogen resulted in a retention at the origin of 91.3% of the total radioactivity in the single batch which was tested.

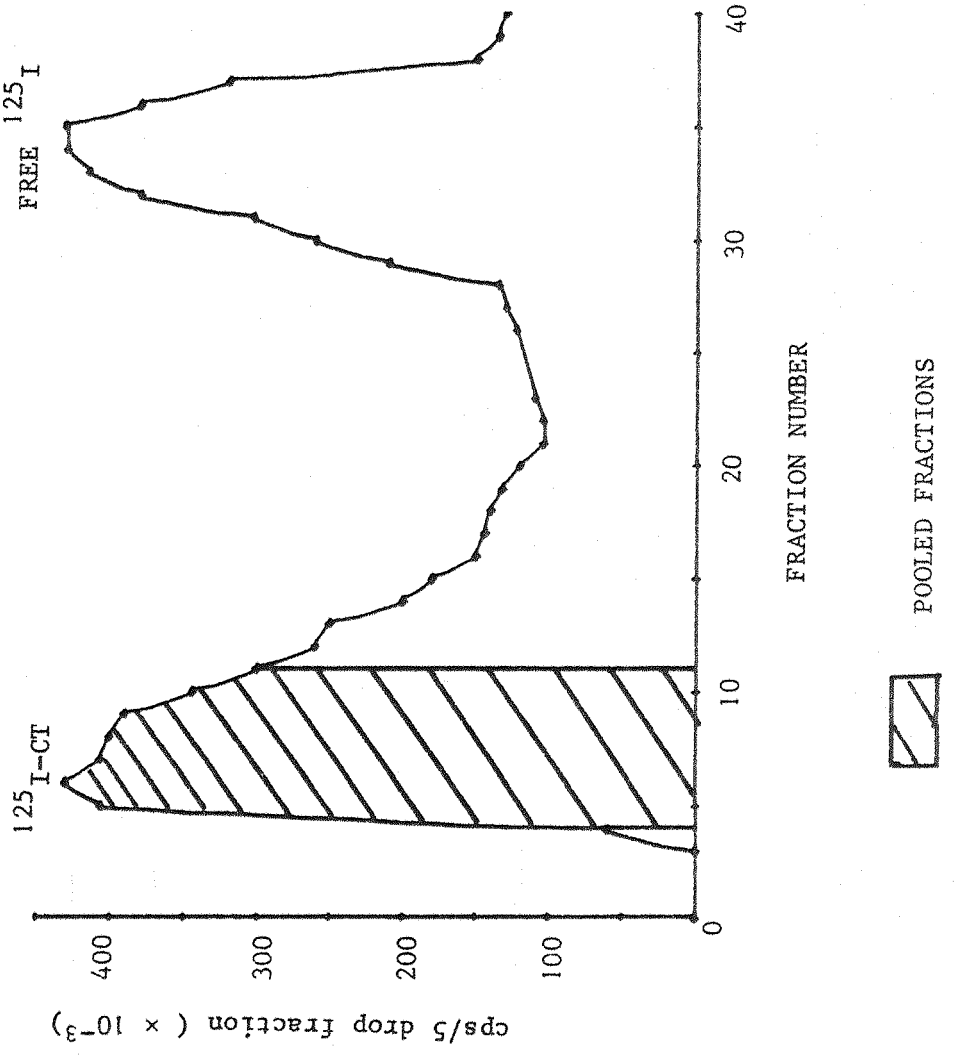
(3) Antisera Titrations

A series of antibody dilutions were incubated with a constant (10,000 cpm) amount of ^{125}I-CT in an incubation mixture containing the following

50 μl Trasylol$^{(R)}$ (A kallikrein-trypsin inhibitor - Bayer Pharmaceuticals)
50 μl antiserum
50 μl ^{125}I-CT
100 μl buffer

The buffer used in the assay, and for all dilutions of antiserum, ^{125}I-CT, standards and samples consisted of 0.05 M phosphate containing 2 g/l BSA, 0.2 g/l thiomersal and 10 m-moles/l EDTA.

FIG. 2.1 Elution profile of a Sephadex G-25 Column used to separate ^{125}I-CT and ^{125}I



An antibody dilution which bound 40-60% of the total counts after four days incubation at 4°C was used in further assays and was found to be 1:400 i.e. a final dilution of 1:2000. The titre of antibody reached a maximum 10 weeks after the intra-dermal injection of sSCT in the best rabbit and sufficient plasma (10 ml) was removed and stored at a dilution of 1:10 in 1 ml aliquots under liquid nitrogen and this stock of antiserum was used in all CT assays reported in this thesis.

(4) Incubation Procedure

The following solutions were added to LP2 polystyrene tubes, mixed and incubated at 4°C for 24 h:

50 µl Trasylol
50 µl 1:400 dilution of antiserum (or buffer)
50 µl buffer
50 µl standard sSCT, sample or buffer (zero CT)

At the end of 24 h a further 50 µl buffer was added containing approximately 10,000 cpm of ^{125}I-CT and the incubation continued for a further 3 days. This "non-equilibrium" approach i.e. the late addition of the iodinated hormone was adopted since it greatly increased the sensitivity of the assay. Standard amounts of sSCT from 0 to 1000 pg/tube were used and each standard or sample was assayed in triplicate and included duplicate, 'no-antibody' controls. The latter were included to assess non-specific binding of ^{125}I-CT. Plasma samples were assayed undiluted and also diluted 1:1 with the RIA buffer.

Incubation damage due to the presence of plasma was assessed by incubating a standard amount of iodinated hormone with various volumes of rat or 3-day old chick plasma (up to 100 µl) and under the conditions used was found to be negligible.

(5) Separation of bound and free hormone

In early experiments separation of bound and free hormone was achieved by two methods.

(a) Double-antibody. At the end of the incubation 50 µl goat anti-rabbit antiserum was added to each assay tube and allowed to incubate for a further 24 h. The precipitated double antibody complex was filtered on a Whatman GF/C glass-fibre paper (2.5 cm) soaked in a mixture of 0.05 M phosphate buffer pH 7.4 and Horse

Serum No. 5 (Wellcome Reagents Ltd., Beckenham, England; 1:1 vol/vol) maintained at 4°C, using a milipore filter holder under suction. Each tube was washed twice with 0.5 ml of the phosphate/serum solution and these washings were passed through the same filter paper. Finally the filter papers were wrapped in a small piece of aluminium foil and the radioactivity counted. Unfiltered tubes and no-antibody controls were also counted to give total and wash blank readings respectively and the percentage of iodinated hormone specifically bound to the goat-rabbit antibody complex determined from the following equation,

$$\% \text{ bound} = \frac{(\text{Test cpm} - \text{Wash Blank cpm}) \times 100}{\text{Total cpm}}$$

(b) Charcoal

A solution of the RIA buffer was kept at 4°C, stirred with a magnetic stirrer and 10 g/l of activated charcoal was added. To each assay tube was added 0.4 ml of this charcoal suspension and the tubes centrifuged at high speed for 1 min. The supernatant, which contained the rabbit-antibody-^{125}I-CT complex was removed under suction and the precipitated charcoal, containing free ^{125}I-CT and a small amount of free ^{125}I, was counted without removal from the assay tube. No-antibody and total cpm were also determined and the percentage of ^{125}I-CT specifically bound to the rabbit-antibody was determined from the following equation,

$$\% \text{ bound} = \frac{(\text{CB cpm/No Ab} - \text{CB cpm/Ab}) \times 100}{\text{Total cpm}}$$

CB = charcoal bound

No Ab = no-antibody controls (to correct for non-specific binding)

Ab = test samples with antibody

When the above separation methods were used in two standard curves (over the range of 0-1 ng "cold" sSCT) it was found that the curves were virtually superimposable, except that the charcoal values of % bound compared to the double antibody method were all approx. 4% greater. This was most probably due to the binding of a small amount of free ^{125}I and/or incomplete precipitation of the rabbit antiserum by the goat-anti-rabbit antiserum. The charcoal method of separation, being much

faster and much less expensive, was therefore used routinely in all RIAs referred to in this thesis.

(6) Standard Curve and Cross-reaction

Fig. 2.2 shows a composite standard curve in which each point is the mean ± S.E. of the mean from three successive standard curves determined at 2, 4 and 6 weeks after the iodination of sSCT. The curve was very reproducible during this period and was linear within the range 50-500 pg/tube. With a sample volume of 50 µl this represents an absolute sensitivity of 1 ng/ml plasma, although in some assays 100 µl plasma samples were used, i.e. increasing the absolute sensitivity to 0.5 ng/ml. Each concentration of sSCT was assayed in triplicate and in addition in two tubes containing no antibody to determine the non-specific binding.

Fig. 2.3 represents the results obtained when different concentrations of sSCT or different volumes of chick embryo plasma (always made up to a total volume of 50 µl with buffer) were assayed. The two displacement curves produced are clearly parallel and confirm the good cross-reaction between salmon and chicken calcitonins reported by Cutler et al (1974).

(7) Recovery

When different amounts of sSCT were mixed with various values of chick embryo plasma (of known CT concentration), calculated and RIA determined values for the final CT concentrations agreed very well. A house standard, consisting of pipped embryo plasma of known CT concentration (4.55 ng/ml plasma), was stored in multiple vials under liquid nitrogen and a sample was assayed in each RIA as a check for between-assay variation. In a total of 8 assays the mean and S.E. of the mean of the CT content of this standard was 4.62 ng/ml ± 0.13 with a range of 4.38 - 4.73 ng/ml.

(8) Calculations

The regression line of the linear portion of each standard curve was determined from a computer program and took the form of the following equation:

FIG. 2.2 Standard curve for the RIA of chicken calcitonin

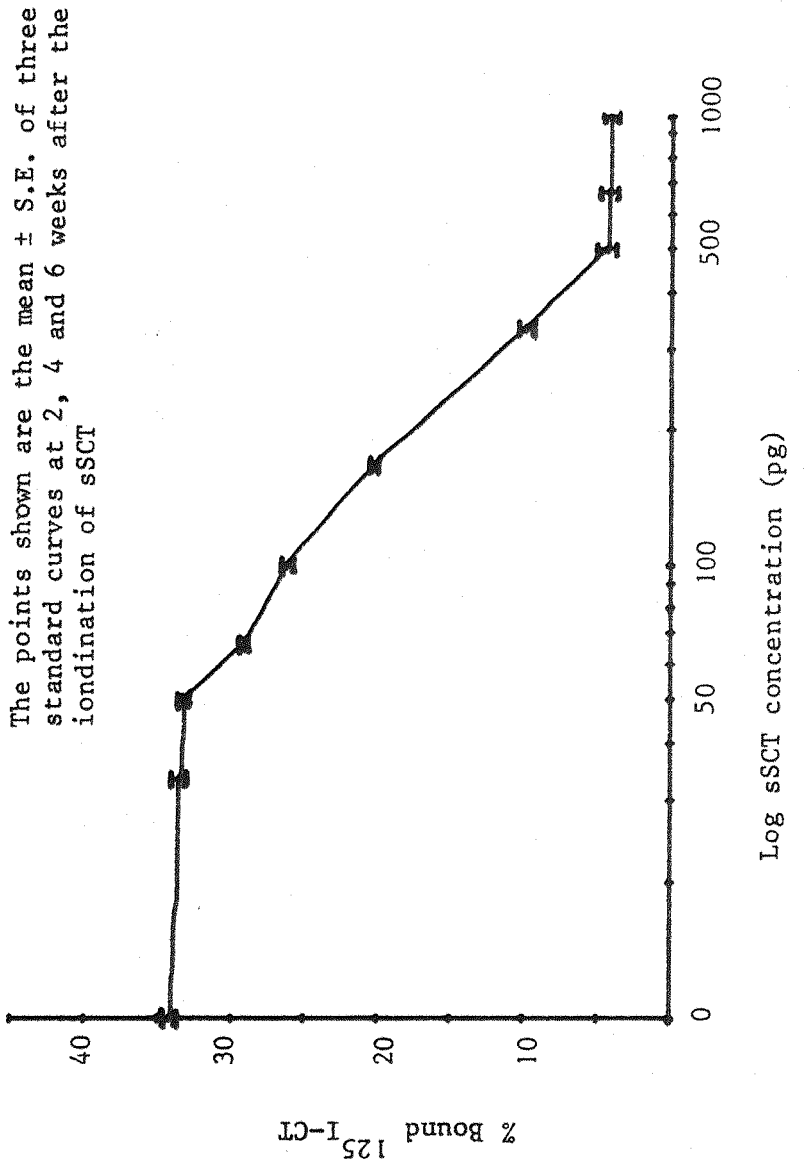
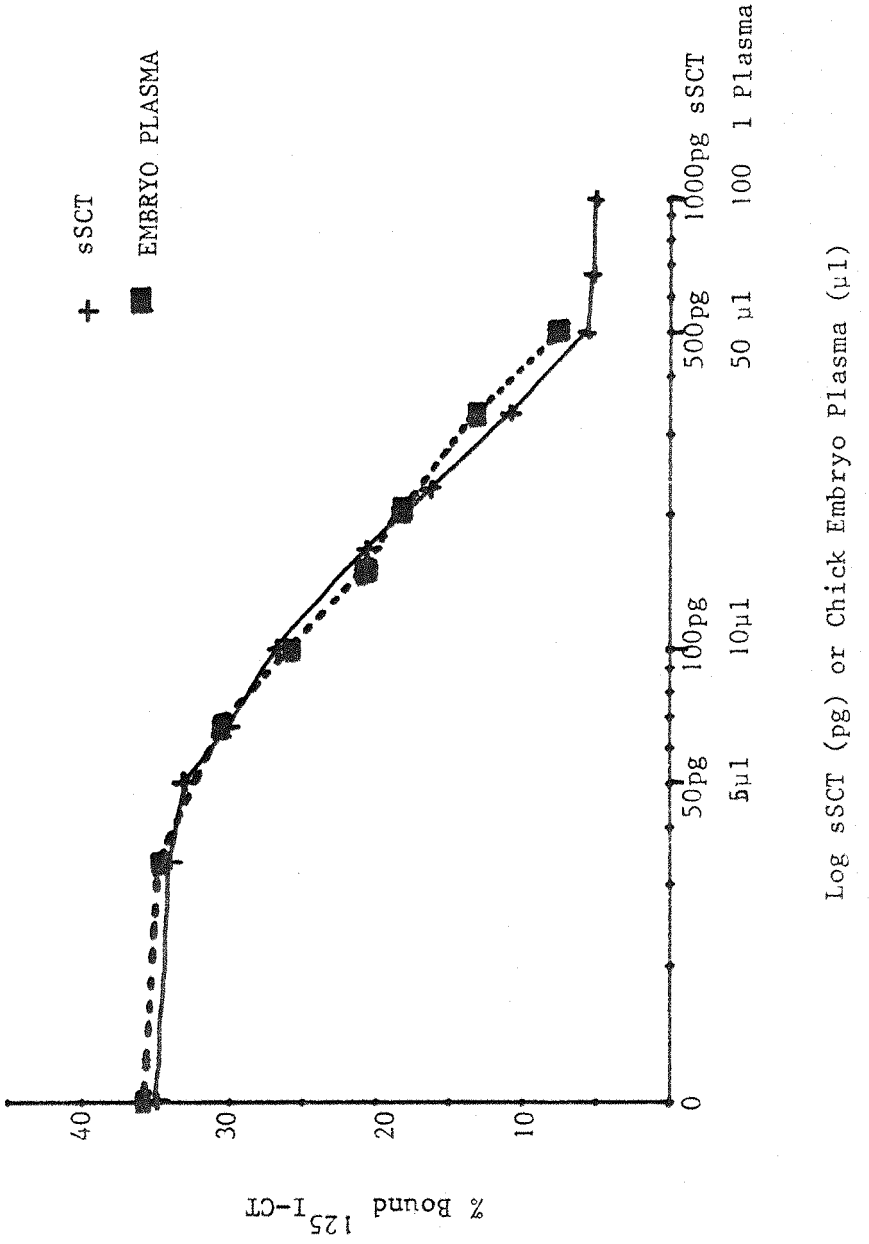


FIG. 2.3 Cross-reaction of sSCT and Chick Embryo Plasma



$$y = A\ E^{BX}$$

where y = % Bound ^{125}I-CT and X = sSCT concentration and A and B were the variable parameters

Thus for an unknown sample

$$\text{CT concentration} = \frac{\log_e \frac{y}{A}}{B}$$

Inserting the value of y (% bound ^{125}I-CT for an unknown sample) into a suitable program gave the result of each sample assay in terms of ng/ml sSCT equivalents.

CHAPTER 3

EXPERIMENTAL

An investigation of the normal events related to the calcium metabolism of the chick embryo.

SECTION 1

The egg shell serves as the major source of calcium for the avian embryo during development. Calcium is removed from the inner surface of the shell and transferred across the ectodermal cell layer of the chorio-allantoic (C-A) membrane to the circulation of the embryo (Terepka, Coleman, Garrison and Spataro, 1971). The process of shell resorption begins on the tenth or eleventh day of incubation and calcium is accumulated rapidly by the embryo for bone formation between the thirteenth or fourteenth day of incubation and hatching (Johnston and Comar, 1955). Studies on the ultrastructure of the parathyroid glands (Narbaitz, 1972) and ultimobranchial bodies (UB) (Stoeckel & Porte, 1969) of chick embryos indicate that these endocrine glands are active during the latter half of incubation but the relative importance of parathyroid hormone (PTH) and calcitonin (CT) in the control of calcium metabolism during the embryonic period is not clear. Vitamin D however, is known to be essential for normal development in the chick embryo (Hart, Steenbock, Lepkovsky, Kletzien, Halpin and Johnson, 1925).

The experiments reported in this section were designed to investigate the normal events relating to the calcium metabolism of the chick embryo that occur during the last week of incubation.

Experiment 1

Changes in the plasma concentration of calcium, magnesium and inorganic phosphate

During the last week of incubation about 125 mg of calcium is transferred from the egg-shell to the embryo and yolk-sac. We wished to assess the ability of the chick embryo to control the total plasma concentration of calcium in the face of this massive transfer of calcium. Previous experiments were inconclusive in that the changes in

plasma calcium in the chick embryo reported by Taylor (1963) were contradicted by Stewart and Terepka (1969). We required to define more accurately the normal levels of calcium at different stages of incubation, in order to correlate any such changes with the levels of CT in the late chick embryo reported by Taylor and Lewis (1972) and confirmed by Cutler, Habener, Dee and Potts (1974). The interrelationship between the metabolism of calcium and phosphate, and the effects of CT upon plasma inorganic phosphate suggested that we might usefully examine the plasma inorganic phosphate concentrations at the same time. Similarly, magnesium has been shown to have effects upon CT secretion rates and the high levels reported by Taylor (1963) warranted further investigation.

EXPERIMENTAL

Fertile eggs were removed daily during the period between the thirteenth and twentieth day of incubation and the embryos bled either from the umbilical artery (days 13-16 only) or from the exposed heart. On day twenty, the eggs were examined at half hourly intervals to identify the onset of pulmonary respiration and pipping. Blood samples were collected at each of these two stages (and also from day 20 eggs still respiring entirely through the shell), at hatching and from one day old chicks. All pulmonary respiring embryos and chicks were anaesthetised with diethyl ether before bleeding. Plasma samples were analysed for calcium and magnesium by atomic absorption spectrophotometry and inorganic phosphate by the method of Chen, Toribara and Warner (1956). The length of the third toe was measured and used to check the stage of development of the chick embryo (Hamburger and Hamilton, 1951). Any embryo who's third toe length fell outside the limits for each particular stage was rejected.

RESULTS

The results are shown in table 3.1 and Figs. 3-1 and 3-2.

The concentration of plasma calcium remained constant at 2.42 - 2.45 m-mole/l between days 14-16 after which it increased rapidly reaching a plateau at 2.61 m-mole/l which was maintained from days 18-20. At hatching (day 21) the mean level fell to 2.55 m-mole/l and one day later it was 2.52 m-mole/l.

TABLE 3.1

Concentrations (m-mole/l) of calcium, magnesium and inorganic phosphate (as phosphorus) in the plasma of chick embryos during development (Mean values* and standard errors with numbers of samples in parentheses)

Days of incubation	Calcium Mean	Calcium SE	Magnesium‡† Mean	Magnesium‡† SE	Phosphorus‡‡ Mean	Phosphorus‡‡ SE
13	-	-	1.13	0.02(11)	2.11^{a}	0.07(11)
14	2.428^{a}	0.010(25)	1.16	0.02(13)	2.45^{b}	0.06(13)
15	2.415^{a}	0.012(20)	1.13	0.02(14)	2.42^{b}	0.06(14)
16	2.450^{a}	0.020(24)	1.06	0.03(8)	2.45^{b}	0.07(8)
17	2.545^{b}	0.013(25)	0.98	0.02(13)	2.27^{ab}	0.06(13)
18	2.588^{c}	0.015(25)	0.97	0.04(6)	1.93^{c}	0.09(6)
19	2.585^{c}	0.010(45)	0.89	0.03(7)	1.96^{c}	0.08(7)
20A†	2.615^{c}	0.020(32)	-	-	-	-
20B†	2.608^{c}	0.015(36)	0.93	0.03(8)	2.11^{a}	0.07(8)
20C†	2.615^{c}	0.013(35)	0.90	0.03(10)	1.66^{d}	0.07(10)
21†	2.548^{b}	0.018(30)	0.88	0.02(11)	1.52^{d}	0.07(11)
22†	2.515^{b}	0.015(38)	0.71	0.02(12)	1.09^{c}	0.06(12)

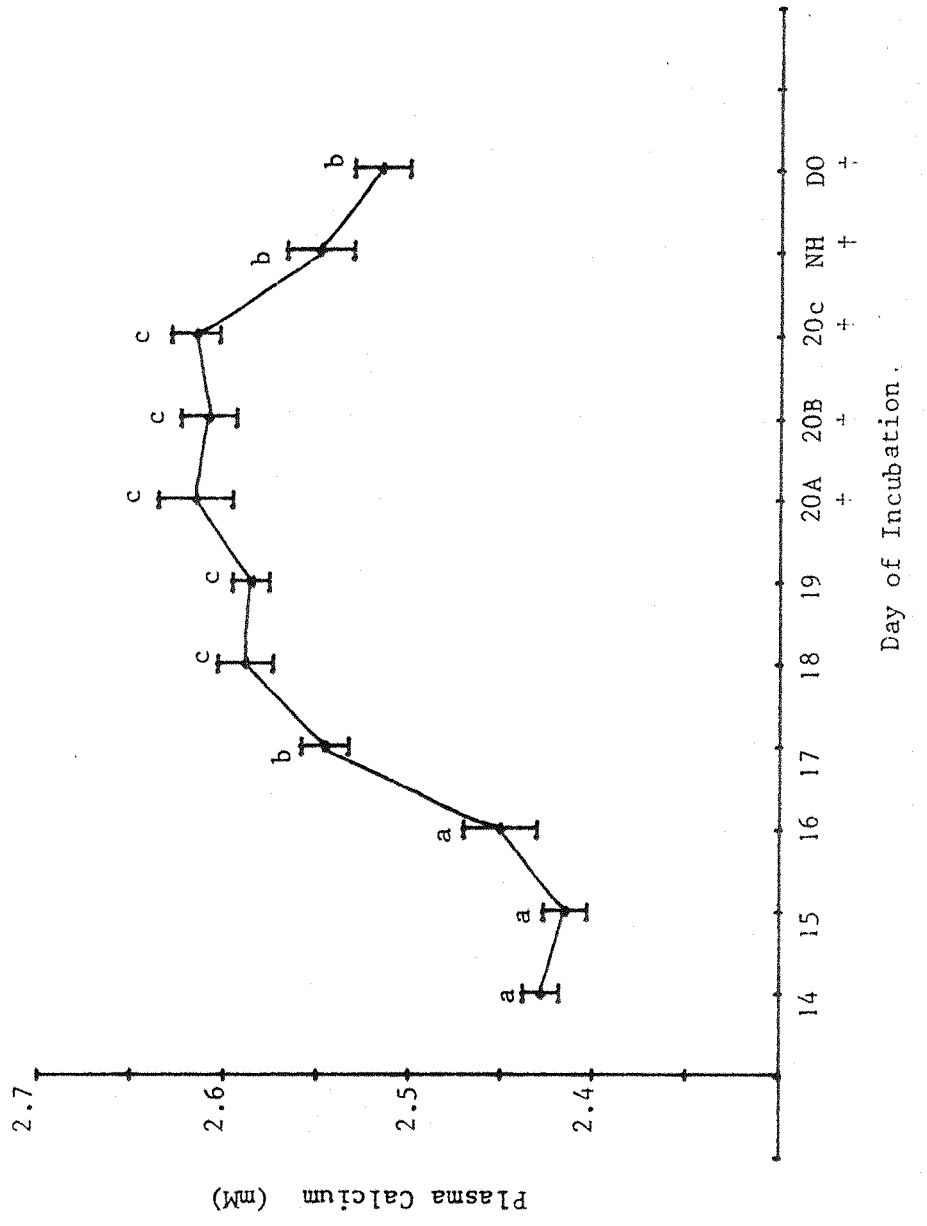
* Means with different superscripts differ significantly from one another ($P < 0.05$, t-test).

† 20A shell respiration, 20B lung respiration, 20C shell "pipped", 21 newly hatched, 22 day-old.

‡‡ Based on physiological age as determined by length of third toe up to day 19 (Hamburger & Hamilton, 1951).

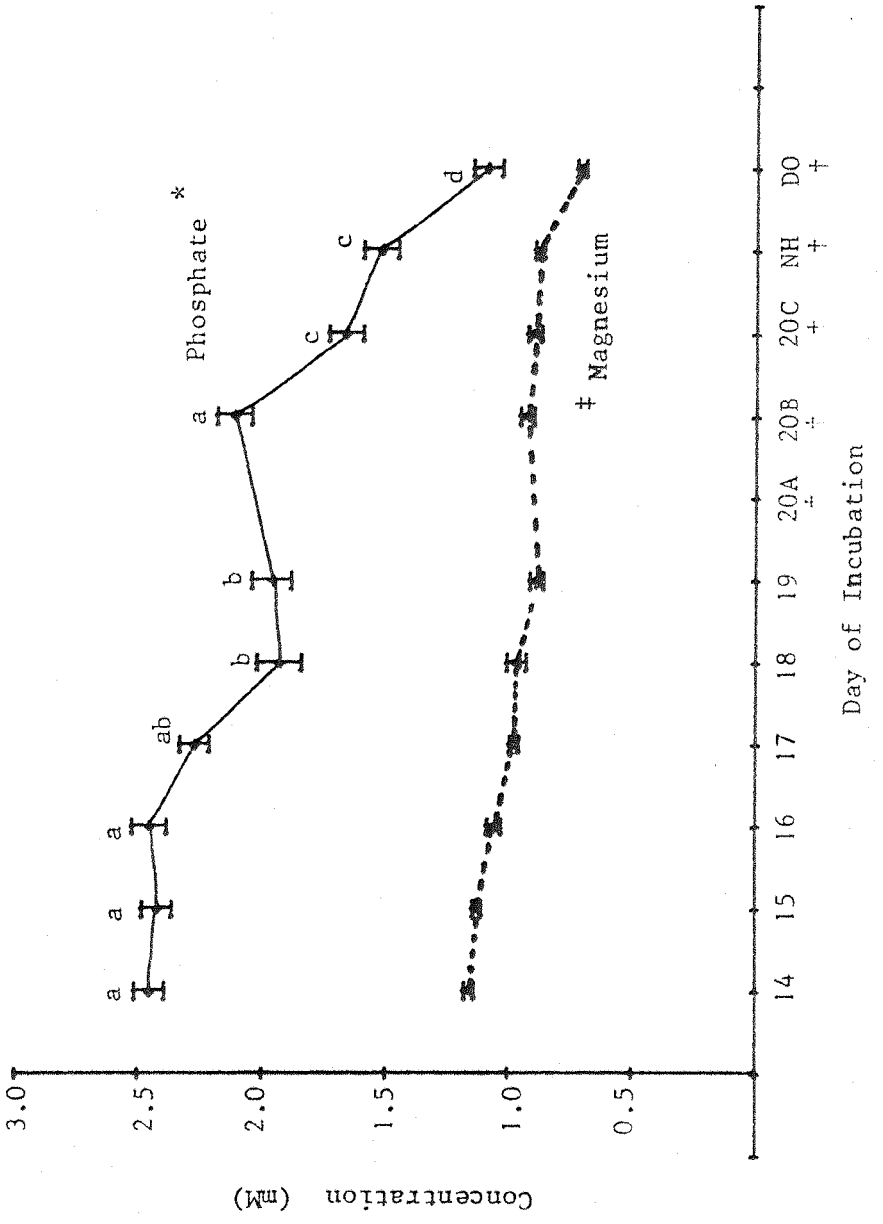
Magnesium values were subjected to regression analysis (see text).

FIG. 3.1 Changes in Plasma Total Calcium Concentration in the Chick Embryo during Incubation



+ 20A shell respiration, 20B Lung respiration, 20C shell 'pipped'
NH newly hatched DO one day old
Means with different superscripts differ significantly from one another ($P < 0.05$, t-test)

FIG. 3.2 Changes in Plasma Magnesium and Inorganic Phosphate in Chick Embryos during Incubation



† 20A, 20B, 20C, NH and DO as in Fig. 3.1

*Means with different superscripts differ significantly from one another ($P < 0.05$, t test)

‡ Magnesium values were subjected to regression analysis (see text)

The mean plasma concentration of magnesium fell steadily from 1.13 m-mole/l on day 13 to 0.88 m-mole/l at hatching, with a further fall to 0.71 m-mole/l one day later. The regression equation expressing this trend was

$$Mg = -0.045d + 1.763$$

where Mg = concentration of plasma magnesium (m-mole/l)
d = day of incubation.

This regression was highly significant ($F = 60.72$, $P < 0.001$).

The concentration of plasma inorganic phosphate fell in a series of stages from 2.4 m-mole/l on days 14-16 to 1.9 m-mole/l on day 18-19 and 1.5 m-mole/l at hatching with a transitory rise to 2.1 m-mole/l at the onset of pulmonary respiration. In the one day old chick the mean value had fallen to 1.09 m-mole/l.

Experiment 2

Changes in plasma calcitonin concentration in the chick embryo.

In 1972, Taylor and Lewis investigated the possibility that calcitonin may be involved in the control of plasma calcium homeostasis in the chick embryo. Using the bioassay of Sturtridge and Kumar (1968), and pooled plasma samples from embryos and young chicks of the same age, they found very high levels of CT, culminating in a peak value of 11,000 mU/l specifically at the time of pipping. The development of a radioimmunoassay (RIA) for chicken CT enabled this experiment to be repeated using individual embryo samples.

EXPERIMENTAL

Embryos and newly hatched and 1 day old chicks were bled as described in Experiment I. Individual embryo plasma samples (up to 0.6 ml) were stored under liquid nitrogen prior to the assay of CT. The RIA was performed on undiluted plasma and also upon the same sample diluted with an equal volume of RIA buffer. Three 50 µl and two 50 µl aliquots were used for the test and blank (no antibody) tubes respectively. The average CT concentration from the undiluted and diluted plasma was taken as the mean unless the diluted plasma fell below the limits of the assay, in which case the concentration determined in the undiluted plasma was accepted.

RESULTS

The results are shown in table 3.2 and Fig. 3-3. The results are expressed as ng/ml equivalents of synthetic salmon calcitonin (sSCT) and in mU/l. The latter values were calculated from a potency of 4790 MRC U/mg for the sSCT used. The bioassay results from Taylor, Balderstone and Lewis (1975), and the RIA results of Cutler et al (1974) are included in table 3.2 for comparison. Cutler's results have been recalculated in terms of mU/l, based upon a quoted potency of 2700 U/mg for the sSCT used in their assay.

Calcitonin was first detectable on day 17 of incubation and remained at detectable levels until one day after hatching. It was no longer detectable in two day old chicks. The CT reached a plateau level of 2.9 ng/ml on day 18 and 19 and in 20 day embryos prior to the onset of pulmonary respiration (20A). A rapid rise to a peak value of 5 ng/ml in pipped embryos was followed by a sharp fall to 2.7 ng/ml in newly hatched chicks. The peak value at pipping was significantly different by a Students t-test from the values found at stage 20B and at hatching, ($P < 0.001$).

Compared to the bioassay results the concentrations at each stage were higher when measured by RIA, although the overall pattern of change was the same, i.e. a rise from day 17 to a peak value at pipping followed by a rapid fall. The RIA results of Cutler et al. were also very similar, although 20C embryos, considered by us to be a distinct developmental stage, were grouped together with newly hatched chicks, (personal communication).

The detection limit of our RIA was 50 pg/tube or 1 ng/ml when 50 µl of plasma was used in the assay (equivalent to 4700 mU/l). The detection limit of the bioassay was 500 mU/l. The level of calcitonin remained undetectable prior to day 17 and in 2 day old and older chicks (< 2350 mU/l) when the RIA sensitivity was doubled by increasing the volume of plasma per tube from 50 to 100 µl.

DISCUSSION

The levels of calcitonin correlate well with the changes in plasma calcium concentration in that a mild, but significant, hypercalcaemia exists in the embryo whenever CT is detectable. However, the level of plasma calcium remained virtually constant from day 18 until pipping,

TABLE 3.2

NORMAL LEVELS OF CALCITONIN IN THE CHICK EMBRYO

Day of incubation	RIA ng/ml	RIA mU/l	Literature*	Bioassay mU/l
16	ND	ND		ND
17	1.572 ± 0.054(3)	7529		1060
18	2.908 ± 0.275(3)	13929	2160	1275
19	2.880 ± 0.206(5)	13795	3410	2002
20A	2.719 ± 0.042(5)	13024	{8180}	2712
20B	3.755 ± 0.213(7)	17986		4744
20C	5.002 ± 0.254(6)	23959	{8630}	11057
NH	2.728 ± 0.268(6)	13067		1412
DO	1.848 ± 0.021(3)	8851	11070	ND
2DO	< 0.75	-		-

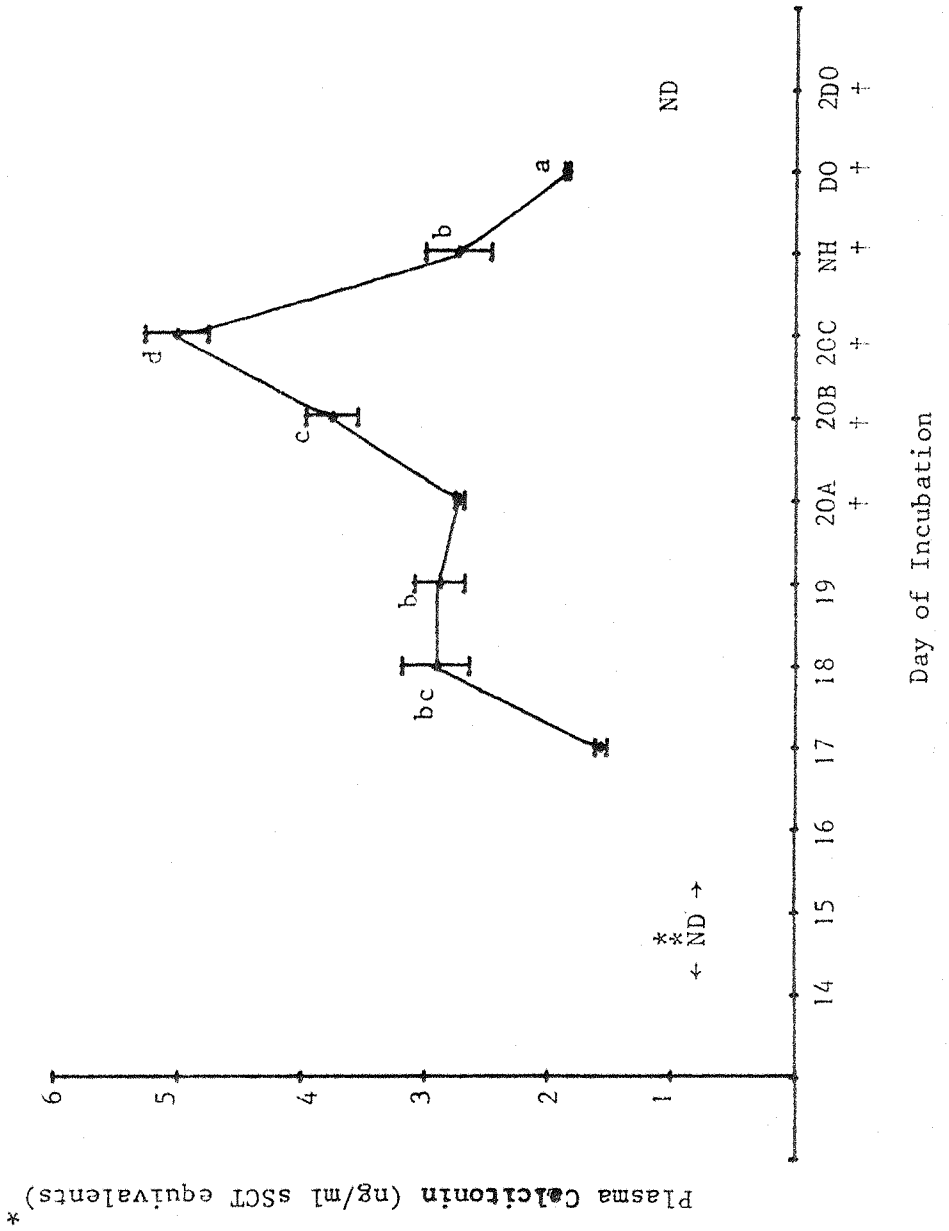
ND - not detectable

* Results from Cutler, Habener, Dee and Potts (1974) recalculated in terms of mU/l.

20A shell respiration, 20B lung respiration, 20C shell "pipped"

NH newly hatched, DO one day old

FIG. 3.3 Changes in Plasma Calcitonin Concentration in the Chick Embryo during Incubation



† 20A, 20B etc. as in Fig. 3.1

* Means with different superscripts differ significantly from one another ($P < 0.05$, t-test)

** ND - not detectable (< 0.75 ng ml)

a period during which the plasma CT increased from 2.9 to 5 ng/ml. It would seem unlikely that a change in plasma calcium concentration alone could have triggered the large release of CT that must have occurred on day 20. Both increases in plasma magnesium and inorganic phosphate have been shown to stimulate the release of CT, but the levels of these ions are falling or constant at the same time that plasma CT is continuing to rise. However, the absolute concentrations of both magnesium and inorganic phosphate are high compared to a young chick and the possibility exists that the UB glands do not respond to these ions until they are more fully developed. Further, Cutler et al. (1974, 1977) have shown that the amount of CT present in the UB gland is very low in young embryos but increases rapidly from day 16 onwards, which might suggest that the effect of high magnesium and inorganic phosphate will not be apparent until the amount of CT available for release has increased. The ability of these workers to detect a fall in the total amount of CT in the UB glands during the time at which plasma CT levels are high suggests that the UB's have a relatively low biosynthetic capacity, so that the requirement for a store of CT to be available before the UBs can respond significantly to any stimulus, becomes apparent.

The resulting actions of the secretion of CT can only be speculative. The changes in plasma calcium and magnesium are small between day 20 and hatching but the concentration of plasma inorganic phosphate is almost halved (2.1 to 1.1 m-mole/l) from stage 20B to one day old chicks. It could however be argued that the fall in phosphate is merely a reflection of its utilisation, in bone formation, at a time when the available phosphate stores are being depleted. The major source of phosphate is the yolk-sac, and the total amount of phosphorus in the yolk falls rapidly from 134 mg at day 14 to 61 mg in day 20 embryos, whilst the total phosphorus in the bone increases from 8 mg to 56 mg over the same period (Romanoff 1967)

The main roles for CT that have been suggested are the protection of the skeleton from excessive resorption and the prevention of hypercalcaemia, particularly post-prandial hypercalcaemia. There is little evidence to suggest that the embryo is at risk from either of these dangers although the absence of any marked hypercalcaemia may suggest that CT has successfully combated a hypercalcaemic challenge, possibly from parathyroid hormone. Copp et al (1970) have shown that infusion of calcium

into a young anaesthetised cockerel resulted in an increase in both plasma calcium and CT. If the cockerel was ultimobranchialectomised during this infusion then the plasma calcium rose a great deal further. This was a convincing demonstration that CT can exercise a restricting role. The ability of CT to act upon normal levels of plasma calcium in the chicken is disputed. Gonnerman, Breitenbach, Erfling and Anast (1972) could find no effect of chicken UB extract upon plasma calcium concentration in the chicken whether or not the parathyroid glands were present. Kraintz and Intscher (1969) however, reported that CT could reduce a normal plasma calcium level if the parathyroid glands were first removed, and they suggested that the failure of CT to act in intact chickens was due to a rapid compensatory action of PTH.

The possible effects of anaesthesia should be considered. Only pulmonary-respiring embryos were anaesthetised and if there was an effect of anaesthesia it would presumably either stimulate or inhibit CT secretion, not both. Since the plasma CT rose from stages 20A to 20C and fell from stages 20C to 22 it appears unlikely that the observed changes in CT concentration were due to anaesthesia.

The location of the peak value of CT specifically at stage 20C is of considerable interest. The pipping stage itself is primarily associated with the development of embryonic pulmonary respiration. The major physiological changes, which take place during this period, are in the gaseous composition of the blood. At stage 20A, while the embryo is still breathing entirely through the shell the pH of the plasma is low (pH 7.39), the P_{CO_2} falls to 38.1 mmHg and the P_{O_2} rises to 40.9 mmHg (Freeman and Misson, 1970).

A further possibility that should be considered, therefore, is that the stimulus to CT secretion may be either increasing hypoxia or increasing respiratory acidosis, both of which are alleviated at pipping when the embryo first starts to breathe normal air. These stimuli may act by way of the autonomic nervous system direct or by releasing catecholamines. The UB glands of chickens are well innervated by fibres from the vagus and to a lesser extent from the recurrent nerve and the sympathetic system (Dudley, 1942; Hodges, 1970). Care and Bates (1973) have demonstrated an increased rate of CT secretion from the UB glands of geese after electrical stimulation of the vagus. The results of experiments reported by Bates, Bruce and Care (1970) suggest that

catecholamines may stimulate the secretion of CT in the pig by activation of the β-receptors and that this stimulation may be inhibited by the activation of the α-receptors or by blockade of the β-receptors.

The progressive fall in the level of plasma magnesium during the latter stages of incubation is most probably associated with the increase in the percentage of this element in the bone ash which, in the early stages of bone calcification in chick embryos is very low (5.2 mg/g at 14 days compared with 9.0 mg/g at day 17 and 10.9 mg/g at day 20; Taylor, 1963).

Finally, mention must be made of the transient nature of the CT peak. The mean length of time between stage 20B and pipping (20C) is about 9 h (Visschedijk, 1968) and during this short period the plasma CT concentration rose from 3.7 to 5.0 ng/ml. The mean interval between pipping and hatching is about 14 h and during this period the plasma CT fell from 5.0 to 2.7 ng/ml. Whatever the stimulus for the release of CT in the embryos it would appear to increase until pipping and then rapidly decrease.

SECTION 2

Historically, the ability of the chick embryo to utilise shell calcium for the calcification of its developing skeleton has attracted considerable attention. As early as 1918 Delezenne and Forneau suggested that carbon dioxide excreted by the embryo provides an acid condition necessary for changing shell calcium from an insoluble carbonate to a soluble oxide. Much later Terepka, Coleman, Garrison and Spataro, (1971) demonstrated the ability of the chorio-alantoic membrane to actively transport calcium from the shell to the embryonic circulation. The removal of calcium from the shell also serves another essential function, namely to increase the porosity of the shell thus increasing the efficiency of gas exchange across the chorio-allantoic membrane, which serves as a respiratory surface for the developing embryo until just before hatching.

The presence of a yolk-sac in this developing system and the apparent deposition of calcium into this store during the last week of incubation is another factor to be taken into account when considering the movement of calcium from one "compartment" to another. Johnston and Comar (1955) determined the total calcium content of the different components of the egg during the entire incubation period. They also labelled the albumen with ^{45}Ca at the start of incubation and followed the transfer of calcium from the shell and albumen to the embryo and yolk. Their results demonstrated a period of rapid skeletal calcification, beginning at day 13-14, but reaching a maximum rate between days 17-19, after which the rate of calcification began to decrease with increasing age. One point of interest to arise from this work was the suggestion that excess calcium might be deposited in the yolk-sac if the rate of transport by the chorio-allantoic membrane exceeded the rate of utilisation of calcium by the embryo. Studies with radioactive calcium led Nozaki, Horii, and Takei to a similar conclusion in 1954.

The experiments reported in this section were designed to further investigate the normal changes in the total calcium content of the yolk and embryo during development and also to investigate the movement of calcium between different components of the egg.

Experiment 3

Total embryo and yolk-sac calcium during incubation

Although previous authors have agreed upon the total calcium content of the yolk-sac and embryo at different stages of incubation we repeated this work using eggs from the same strain of bird (Ross I)which have been used for all the work presented in this thesis. Between the onset of calcification and hatching, more than 100 mg of calcium are deposited in the bone of the chick embryo. The increase each day from day 14 onwards is not the same and we particularly wished to determine whether or not there was an unusually large rate of calcium deposition towards the end of the incubation period.

EXPERIMENTAL

Fertile eggs of the Ross I strain were incubated and at least five were removed at random from the incubator from day 14 onwards. The embryo and yolk-sac were carefully dried to remove any adhering albumen, placed separately into previously weighed porcelain crucibles and charred over a bunsen flame. The contents were then ashed in a muffle furnace overnight at 600°C. The ash weigh was determined and then the contents of each crucible were dissolved in 5.5 M Hydrochloric acid. From trial studies the total volume was calculated for embryos and yolk-sacs of each age which would give a suitable concentration of calcium for determination by A.A.S.

RESULTS

The results of this experiment are shown in table 3.3 and Fig. 3-4.

The total calcium content of both the embryo and yolk-sac rose progressively from day 14 of incubation until stage 20B. The embryo calcium increased from 17 mg to 90 mg whilst the yolk-sac calcium increased from 21 mg to 59 mg. From 20B onwards the amount of calcium in the embryo continued to increase to a value of 126 mg in the day old chicks whilst the calcium in the yolk-sac fell to 44 mg during the same period of time.

TABLE 3.3

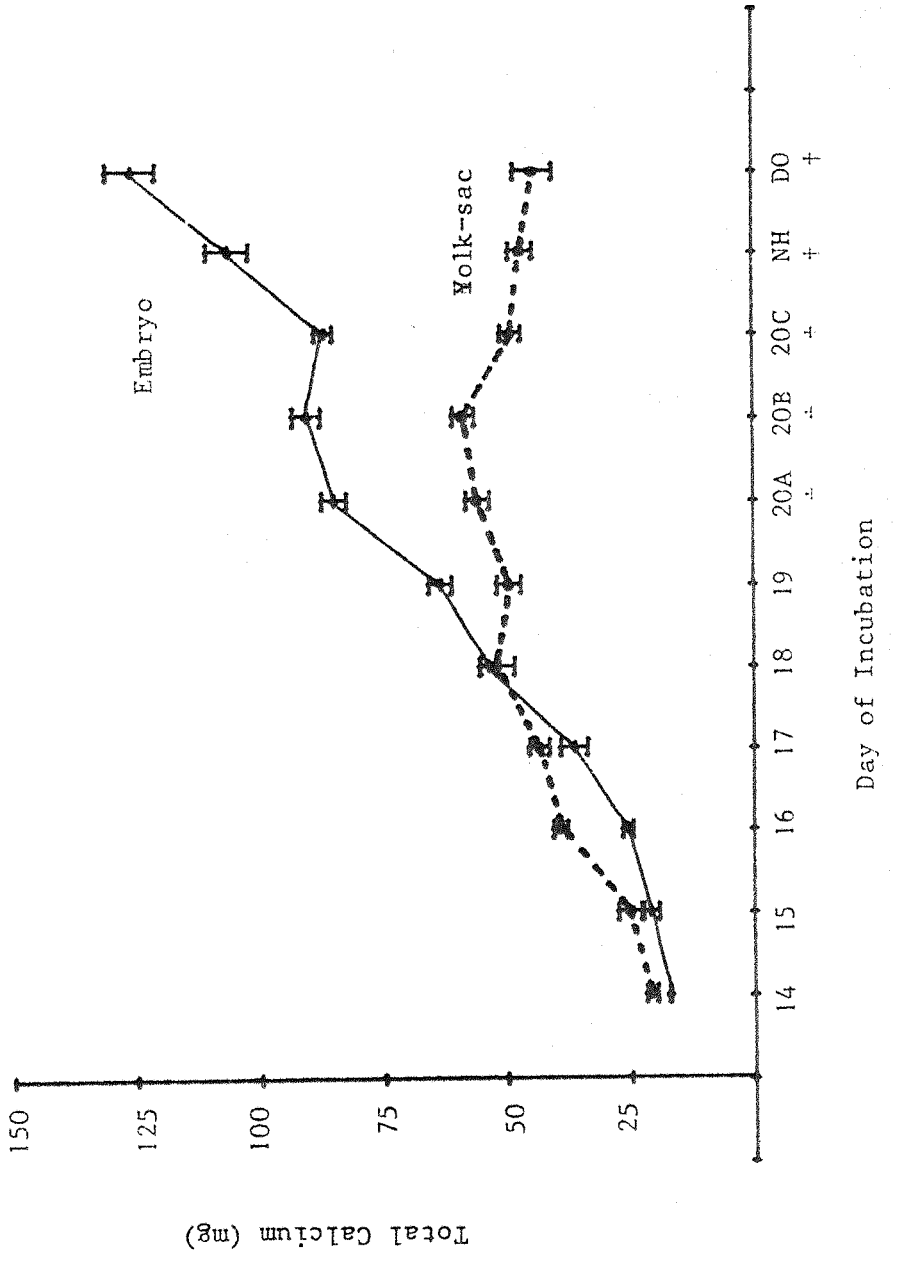
TOTAL CALCIUM IN THE EMBRYO AND YOLK-SAC
(mg)

AGE OF EMBRYO (d)	EMBRYO	YOLK-SAC	N
14	17.2 ± 0.1	20.8 ± 1.1	5
15	21.0 ± 1.9	25.0 ± 2.5	4
16	25.6 ± 1.2	39.1 ± 1.4	5
17	36.3 ± 2.7	43.3 ± 2.0	5
18	53.6 ± 1.8	46.9 ± 3.6	5
19	63.3 ± 2.4	49.4 ± 2.5	5
20A	84.9 ± 2.6	55.8 ± 2.4	5
20B	90.5 ± 2.9	58.6 ± 2.2	5
20C	87.0 ± 1.9	48.9 ± 2.1	5
NH*	106.3 ± 4.3	46.9 ± 2.4	4
DO†	125.9 ± 5.0	44.4 ± 3.9	5

* newly hatched

† day-old

FIG. 3.4 Total Calcium Content of the Chick Embryo and Yolk-sac at Different Stages of Incubation



† 20A, 20B, etc. as in Fig. 3.1

Experiment 4

Distribution of radiocalcium injected into the albumen of chick embryos

Terepka et al (1971) demonstrated the rapid rate of calcium transport which occurs across the chorio-allantoic membrane during the period of rapid bone calcification in the developing chick embryo. They also showed that the back flux of calcium from the serosal, (albumen),side of the membrane was minimal, less than 5% of forward flux, even in the presence of a large downhill calcium gradient. The yolk-sac membrane is also very impermeable to calcium from day 12 of incubation onwards. This is due to the extension of the area vasculosa over the whole of the yolk with the exception of a very small area, the yolk-sac umbilicus, which still retains contact with the albumen (Romanoff, 1960). The impermeability of the yolk-sac to the albumen was also demonstrated by marking the albumen with a variety of stains (Hanan, 1927). The stains appeared in the amniotic fluid, the digestive and respiratory tracts but not in the yolk-sac.

The isolation of the albumen from the chorio-allantoic membrane, (and therefore the shell), and the yolk-sac after day 12 of incubation enabled us to investigate the movement of ^{45}Ca from the albumen to the embryo. The only route available for the transfer of ^{45}Ca to the embryo and yolk-sac after day 12 could be via the intestine. The albumen and amniotic fluid mix prior to being swallowed by the embryo (Romanoff, 1960).

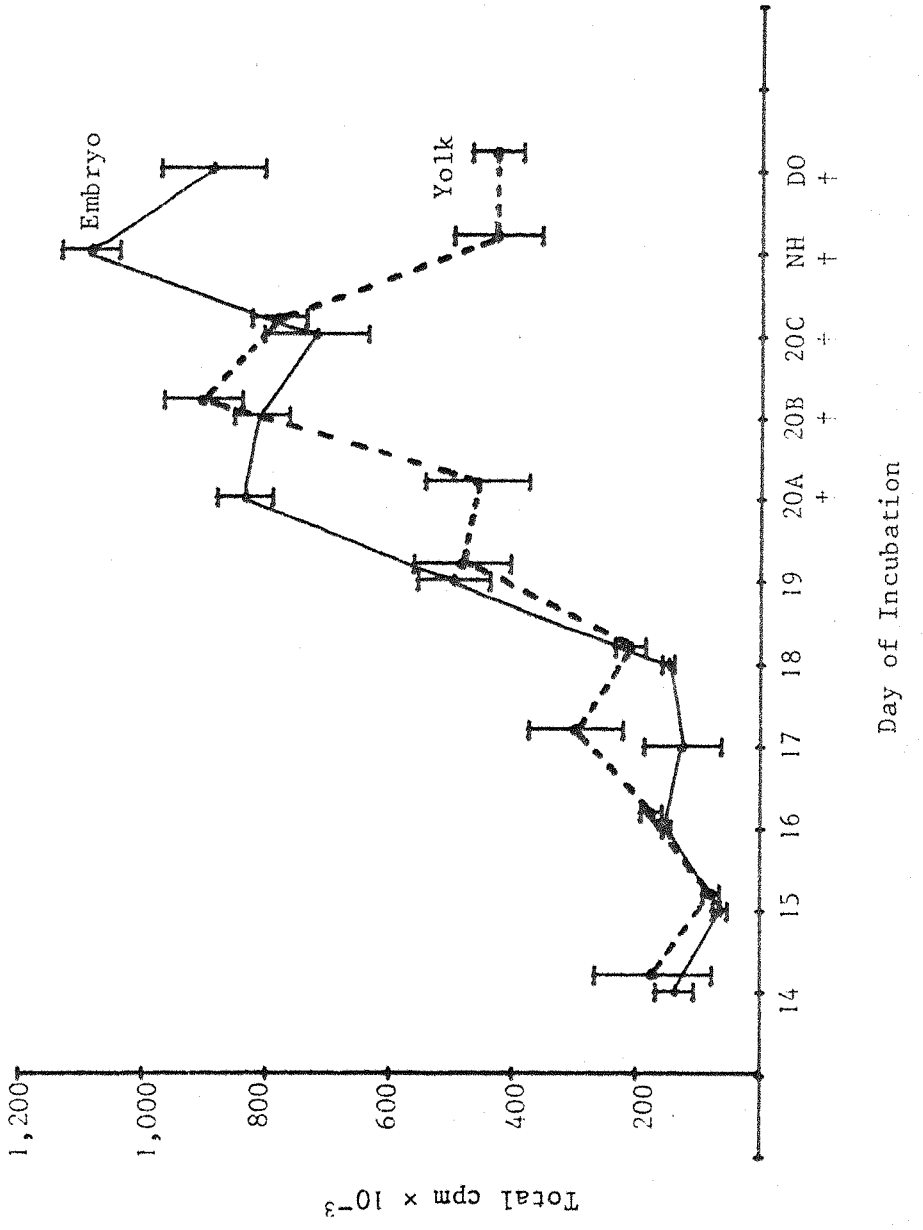
EXPERIMENTAL

On the 12th day of incubation eggs were removed from the incubator and the albumen was injected with 1 μCi of ^{45}Ca in distilled water. From day 14 of incubation and on each succeeding day, including the three physiological stages on day 20, hatching and 1 day old chicks, a random sample, of at least five eggs, was taken and the total ^{45}Ca activity in the embryo and in the yolk-sac was determined.

RESULTS

The results are shown in Fig. 3-5. The total number of counts per minute (CPM) in the embryo remained constant at about 150,000 from days 14 to 18. This was followed by a 6-7 fold increase in the total CPM in the newly-hatched chick. There was a small fall in the total

FIG. 3.5 Distribution of ^{45}Ca Injected into the Albumen of Chick Embryos on Day 12 of Incubation



† 20A, 20B, etc. as in Fig. 3.1

CPM in the 1 day old chicks but this was not significantly different from the newly-hatched value.

The total ^{45}Ca content of the yolk-sac also remained constant from days 14-18, but at a slightly higher level (170,000 CPM). This was followed by a 5-6 fold increase in the ^{45}Ca content until stage 20B (onset of pulmonary respiration). Thereafter the total ^{45}Ca content fell by about 50% to a value of about 400,000 in newly-hatched and 1 day old chicks.

Experiment 5

Distribution of Radiocalcium injected i. v. into chick embryos

Having entered the embryonic circulation, from whatever compartment, calcium is then available for utilisation by the embryo. The entry of calcium into the blood vessels of the chorio-allantoic circulation is probably proceeding at a maximum rate by day 16 of incubation (Terepka et al., 1971) and is independent of the rate of utilisation. Some homeostatic mechanism must be operating in the embryo in order to maintain the relatively constant plasma calcium concentration reported in Experiment 1. Clearly, if the rate of utilisation of calcium in bone formation is less than the rate of entry of calcium into the circulation then calcium must be removed from the circulation to prevent hypercalcaemia. Johnston and Comar (1955), and Nozaki et al (1954) have suggested that the yolk-sac may be used as a pool of calcium into which excess calcium may be deposited. We wished to confirm this possibility by examining the distribution of an intravenous dose of ^{45}Ca to the embryo and yolk-sac. By using an intravenous injection ^{45}Ca could only be deposited in the bone, the yolk-sac, the soft tissues of the embryo or excreted into the allantoic fluid. The shell and albumen should remain unlabelled and interpretation of the results should be unequivocal.

EXPERIMENTAL

Randomly selected fertile eggs were removed from an incubator starting at day 13. The eggs were injected intravenously with a dose of 1 µCi ^{45}Ca in 0.1 ml physiological saline. The injections were carried out using sterile conditions and under a dissecting microscope. Embryos were rejected from the experiment if, on removal of the needle from the vein, any haemorrhage occurred. Twenty-four hours after injection, the embryos were bled either from the umbilical artery or by direct cardiac

puncture, and then the embryos were separated from their yolk-sacs and the total ^{45}Ca content of the embryo, yolk-sac and a 0.1 ml aliquot of plasma was determined as previously described.

RESULTS

The results are shown in table 3.4 and Fig. 3-6. The total CPM found in the embryo at day 16, expressed as the % recovered dose found in the embryo and yolk-sac of each individual egg, was 64.1% and increased progressively to a value of 93.3% in the 1 day old chick. The % ^{45}Ca in the yolk sac fell from 35.9% at day 16 to 6.7% in the day old chick.

The CPM per 0.1 ml plasma remained constant at about 200-250 from days 16 till pipping but then increased slightly but significantly to 270-300 CPM in newly-hatched and 1 day old chicks. Less than 1% of the total dose of ^{45}Ca injected was found in the plasma of the embryos at the time of bleeding.

DISCUSSION

The experiments reported in this section have attempted to investigate some of the movements of calcium within the fertile egg, and to examine in particular the possibility that the yolk-sac can act as a calcium pool, and thus play a role in the homeostasis of plasma calcium in the developing embryo.

The results of experiment 3 confirm the results of Johnston and Comar (1955) but the continued measurement of the total calcium content of the yolk has revealed a fall in calcium after the pipping stage, suggesting that the calcium stores within the yolk-sac are utilised by the embryo in the late stages of incubation and in the first few days in the free-living existence. Generally the 'spare yolk', which remains in the abdomen of the chick at hatching and which is connected to the small intestine by the yolk-stalk, is totally utilised as a first food source for the young chick by the 5-7th day, post-hatching.

Anatomically, the endodermal layer of the yolk sac of the embryo acts as an absorbing surface for nutrients similar to the endodermal layer of the intestines. Towards the end of the incubation period the yolk-sac contents are actively forced into the intestine (Romanoff, 1960) and it is thought that the digestive function of the embryonic

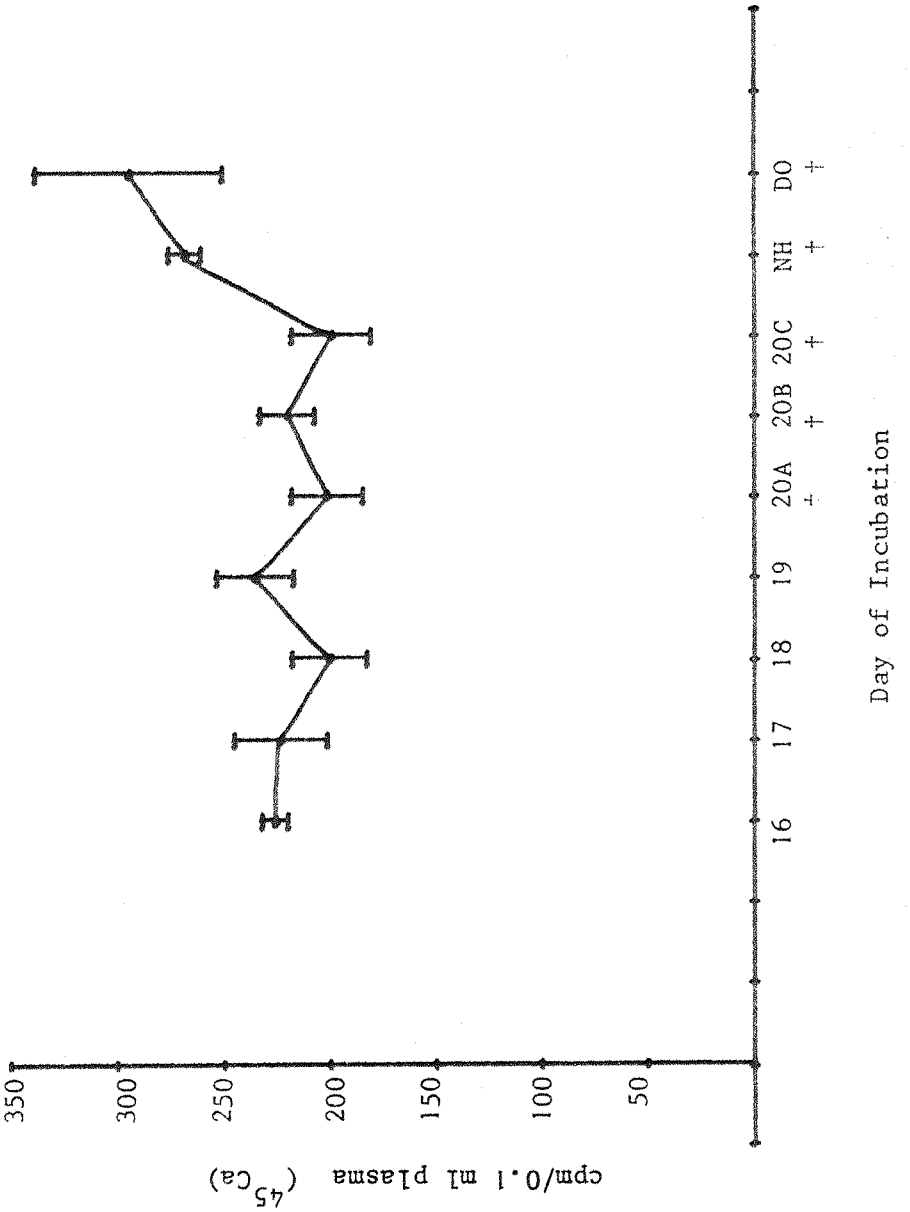
TABLE 3.4

DISTRIBUTION OF AN I.V. DOSE OF 1 μCi ^{45}Ca 24 hrs POST INJECTION

Age of Embryo at bleeding (d)	% Recovered Dose ^{45}Ca in Embryo	% Recovered Dose ^{45}Ca in Yolk-sac	N
16	64.1 ± 2.6	35.9 ± 2.6	4
17	73.6 ± 1.7	26.4 ± 1.7	6
18	77.5 ± 2.2	22.5 ± 2.2	6
19	86.6 ± 1.1	13.4 ± 1.1	5
20A	87.6 ± 0.9	12.4 ± 0.9	6
20B	86.8 ± 1.3	13.2 ± 1.3	6
20C	87.2 ± 1.6	12.2 ± 1.6	6
NH	92.3 ± 0.6	7.7 ± 0.6	4
DO	93.3 ± 1.2	6.7 ± 1.2	4

20A shell respiration, 20B lung respiration, 20C shell "pipped"
NH newly hatched, DO one day old chick

FIG. 3.6 Changes in the Plasma ^{45}Ca Activity after the i.v. Injection of 1 μCi ^{45}Ca 24 h before bleeding



† 20A, 20B etc. as in Fig. 3.1

intestine increases rapidly at this time. The major route for the absorption of the spare yolk of the chick is via the intestine, since the yolk-sac membrane becomes progressively less active and its blood supply is rapidly diminished from the time it first retracts into the abdomen of the embryo.

The results of experiment 4 demonstrate a number of interesting points. First, the amount of ^{45}Ca in both the yolk-sac and the embryo remains constant from days 14-19. What ^{45}Ca is found at these stages is most probably due to direct diffusion of the albumen ^{45}Ca to the yolk-sac via the yolk-sac umbilicus or via small areas of the yolk-sac that have not been fully covered by the extension of the area vasculosa. The embryonic ^{45}Ca at these stages is most probably due to the fact that the embryos, even at these early stages, had swallowed some of the albumen and no attempt was made to separate this ingested albumen from the embryo before ashing.

The second point is that there appears to be an abrupt uptake of albumen ^{45}Ca into both the yolk-sac and the embryo from day 19 onwards. As suggested in the introduction to experiment 4 the only route available for the transfer of the albumen ^{45}Ca to the embryo is via intestinal absorption. Thus it would appear that the ability of the intestine of the embryo to absorb calcium is not present to any appreciable extent until day 19. It is interesting to note that the experiments of Corradino, Taylor and Wasserman (1969) and Corradino and Wasserman (1971) suggested that calcium-binding protein, (CaBP), thought to be a prerequisite for intestinal calcium transport, is not found in the chick embryo until hatching. These results have been confirmed by Moriuchi and De Luca (1974). Clearly, our results show that calcium can enter the embryonic circulation before CaBP is detectable. Most probably the explanation of this discrepancy is due to the fact that histological studies of the embryonic intestine have demonstrated that there are present a number of pinocytotic vesicles, which presumably transport the contents of the intestine, (including ^{45}Ca), to the embryo, without prior digestion or involvement of the usual transporting processes. Certainly it has been shown that the plasma of the chick embryo contains ovalbumen which must have entered the circulation by such a process (Kaminski and Durieux, 1956). The rapid increase from day 19 onwards, occurs at a time when the albumen is being most rapidly swallowed. By the pipping stage virtually all of the albumen and amniotic fluid has been swallowed by the embryo and all of the ^{45}Ca is found either in the

embryo or yolk ash.

The third point of interest is the decline in the ^{45}Ca content of the yolk at pipping and at later stages. The falling ^{45}Ca content of the yolk-sac is very well correlated to a continued rise in the ^{45}Ca content of the embryo. This provides very good evidence that the calcium content of the yolk is utilised by the embryo in the late stages of incubation.

At the onset of pulmonary respiration the blood supply to the chorio-allantoic membrane declines rapidly, and in fact all of the blood vessels are totally constricted by the time of hatching, (36 hours later), and the chorio-allantoic membrane is deteched from the hatching chick and remains within the egg-shell at hatching, (Romanoff, 1960). The C-A and shell membranes become detached from the shell at about day 17 but mammillary cores are always associated with the shell membrane and contain calcareous portions of the eggshell (Tyler & Simkiss, 1959). The decline in the blood supply to the chorio-allantoic membrane is associated with the change to pulmonary respiration, but at the same time the loss of the chorio-allantoic blood supply also cuts off the supply of calcium from the mammillary cores to the embryo. It is precisely at this time that the decline in ^{45}Ca content of the yolk-sac is first apparent.

Experiment 5 provides unequivocal evidence that the yolk-sac does indeed act as a clacium reservoir in the young embryo. Having entered the embryonic circulation the changes in the distribution of ^{45}Ca to the embryo or its yolk-sac are dramatic. On day 14 about one-third of the ^{45}Ca is found in the yolk-sac but the percentage of ^{45}Ca finding its way to the embryo increases rapidly with increasing age, until virtually all the plasma ^{45}Ca is utilised directly in bone formation, and little ^{45}Ca is deposited in the yolk-sac.

Some mechanism for the deposition of ^{45}Ca into the yolk must exist and we believe that this mechanism is a purely physical one and most probably is not dependent upon any hormonal action. The concentration of ionic calcium within the yolk is almost certainly a great deal less than that in the plasma due to the presence of abundant amounts of phospho-proteins within the yolk. This would provide a suitable concentration gradient for the transfer of calcium from the blood to the yolk. The removal of calcium to the yolk would be in competition with

the removal of calcium due to the formation of new bone and presumably some factor involving the relative blood supply to the yolk-sac membrane and to the developing skeleton would influence the relative distribution of plasma calcium to these two compartments. The combined effects of the removal of the shell as a source of calcium and the decline in the blood flow to the yolk-sac membrane, would serve to direct plasma calcium more and more towards bone formation. The effective removal of the source of calcium, i.e. the shell, then requires the utilisation of the yolk stores of calcium, as shown in experiment 4.

The small but significant rise in the CPM of ^{45}Ca in 0.1 m plasma in the newly-hatched and 1 day old chick is interesting and may represent the effect of the removal of ^{45}Ca from the yolk-sac. However the comparatively low counts and the fairly large variation suggest that it would be unsound to rely upon these results as a basis for any argument. The increase in the ^{45}Ca content of the plasma may simply be explained by a fall-off in the rate of utilisation of calcium in bone formation.

CHAPTER 4

The experiments reported in Chapter 3 were all designed to investigate the normal events occurring in the last week of incubation of the chick embryo and the first one or two days post-hatching. The changes in plasma calcium and calcitonin were established and a role for the yolk-sac in calcium homeostasis was suggested. The large movements of calcium from the shell, (or the yolk), to the embryonic circulation and then to the developing skeleton were shown to occur without producing any dramatic changes in plasma calcium concentration. However, the large increase in circulating plasma CT around day 20 suggested that this hormone, well known as a hypocalcaemic agent in mammalian systems, may be playing an active part in the maintenance of a constant plasma calcium concentration.

The physiological role of CT in lower vertebrates is by no means established. In particular, the ability of CT or ultimobranchial extract to produce a hypocalcaemia in the chicken is contested. Kraintz and Intscher (1969) suggested that such a hypocalcaemic effect could be observed, but only if the birds were partially parathyroidectomised. They suggested that the much more rapid action of PTH in birds, clearly shown by Candlish and Taylor (1970), would normally mask any hypocalcaemic action of CT. Calamy and Barlet (1970) observed significant reductions in plasma CT when large doses of porcine CT, (30 units /kg) were infused intravenously over a two hour period in 3-month old cockerels and in laying hens. Other workers (Urist, 1967; Candlish and Taylor, 1970) have failed to demonstrate such an effect. Of particular interest is an experiment performed by Copp, Brooks, Low, Newsome, O'Dor, Parkes, Walker, and Watts (1970). They examined the effect of bilateral ultimobranchialectomy during calcium infusion in a young anaesthetised turkey. The induced hypercalcaemia appeared to be well controlled and associated with an increase in the level of circulating CT. However, UBX resulted in a rapid increase in plasma calcium and a fall, to undetectable levels, in plasma CT. This experiment remains, in our view, the best demonstration that, in the chicken, CT may only play a restrictive role in the control of plasma calcium homeostasis and that the rapid compensatory action of PTH may prevent a fall in plasma calcium concentration below normal levels.

The experiments in this chapter have been designed to examine the effects of exogenous synthetic salmon calcitonin upon various physiological measurements in the chick embryo in an attempt to discover the role of the high circulating levels of CT found in chick embryos just before hatching.

Experiment 6

The effect of exogenous synthetic Salmon Calcitonin in the chick embryo.

Having established that high circulating levels of CT appear in the late chick embryo we wished to establish the resulting actions of CT in this system. In attempting to determine the actions of a hormone there are two basic approaches. The first is to add an exogenous, (or to stimulate an endogenous), supply of the hormone and determine its effect on whatever systems are thought to be involved in the actions of the hormone. The second is to remove the hormone most commonly by removal of the gland responsible for its synthesis and release.

The similarity in biological and chemical properties between salmon and chicken CT in terms of the minimal effective does, duration of the hypocalcaemic effect, (in the rat), and amino-acid composition is well established. (Nieto and Candela, 1972; Nieto, Moya and Candela, 1973; O'Dor, Parkes and Copp, 1969). In all experiments involving the addition of exogenous CT the source was synthetic Salmon CT (sSCT, biological potency, 4790 u/mg). The advantages of using sSCT were as follows. First, by virtue of being synthesised chemically the preparation was homogeneous and free from interfering peptides which are likely to be present in biological preparations. Second, the values for the normal circulating levels of CT in the chick embryo were calculated in terms of "Salmon CT equivalents", since sSCT was used both to raise antibodies and as the standard and iodinated source of hormone in the RIA developed to detect chicken calcitonin. Thus, the dose of sSCT injected into chick embryos could be accurately based on known values for normal, circulating levels.

In this experiment we report the effect on chick embryos of intravenously injected sSCT. As well as the well known effects of CT upon calcium and phosphate metabolism, a number of experimenters have suggested that CT may play a role in salt and water homeostasis.

(Barlet, 1972; Paillard, Ardaillou and Malendin, 1972; Bijvoet, Van der Sluys Veer, Greven and Schellekens, 1972). We have therefore considered this possibility in this experiment.

EXPERIMENTAL

Fertile eggs (Ross I) in the weight range 50-60 g were incubated in a forced draught incubator and transferred to an observation incubator on the nineteenth day. Eggs were selected at random and injected with the following solutions:-

Soln. A 2.5 ml NaCl, (9 g/l), containing 0.025 g Pontamine sky blue and 0.25 g bovine serum albumin.

Soln. B 2.5 ml NaCl, (9 g/l), containing approx. 50 µCi $^{35}SO_4$ and 0, 0.25, 2.5 or 25 µg of sSCT, (activity 4790 u/mg).

Both solutions were kept between 2 and 4°C and solution A was filtered before mixing with solution B. The syringe used for the injection was weighed before and after use for greater accuracy, and the injection volume was 0.1 ml. The doses of CT used were such that 0.1 ml of the mixed solutions contained 0, 5, 50 or 500 ng sSCT and approximately 1 µCi of $^{35}SO_4$. In additional experiments ^{14}C- mannitol was used in place of $^{35}SO_4$ to estimate the extracellular fluid volume. The injected amount of CT was in addition to any already circulating in the day 19 embryos. Based on a plasma volume of approximately 2-3 ml, the lowest dose of CT, i.e. 5 ng, was estimated to increase the circulating levels of CT from 2.9 ng/ml (Experiment 2) to 5 ng/ml, which was the highest circulatory level found naturally (in pipped embryos).

At least four eggs were bled at each of four different times post-injection for each dose of CT, including the zero CT controls. The times of bleeding were 10, 20, 40 and 80 min post-injection, and blood was collected into heparinised syringes. The plasma was divided and analysed as shown below:-

Volume of Plasma	Determination	Method
2 × 100 µl	$^{35}SO_4$ or ^{14}C- mannitol	Liquid scintillation counting in a wide channel
1 × 100 µl	Ca^{++} and Mg^{++}	Atomic Absorption Spectrophotometry

Volume of Plasma	Determination	Method
1 × 100 µl	Inorganic Phosphate	Chen et al (Colorimetric method)
1 × 50 µl	Na^+ and K^+	Integrating Flame Photometry
1 or 2 × 100 µl	Pontamine Sky Blue	Colorimetric: reading absorption at 600 nm

Corrections were made where appropriate for the slight variation of amount injected on a weight basis. The third toe length was measured and any embryo which fell outside the range for day 19 was rejected (Hamburgher and Hamilton, 1951).

RESULTS

The results are shown in tables 4.1, 4.2, 4.3, 4.4 and 4.5.

There were no significant changes in the concentrations of plasma calcium, phosphate or magnesium nor in the plasma volume, at any of the four time-intervals or with any of the doses of CT, compared to control (no CT) values.

The plasma sodium concentrations were not significantly different from control values with the exception of the effect of 50 ng CT at 10, 20 and 40 min post-injection. At this dose and times the plasma sodium concentration was significantly raised above control values.

Small increases in plasma potassium concentration were noted when a dose of 500 ng CT was injected, but the increase from a mean of 6.93 mM in the controls to 8.20 mM in the experimental group was the only one that was significant.

The apparent ECF volume increased with time, due to the slow uptake of $^{35}SO_4$ by the embryo. This resulted in the removal of $^{35}SO_4$ from the ECF and was recognised as an apparent increased dilution of the marker (table 4.4). This dilution was significantly reduced by 5 ng sSCT at 40 and 80 min post-injection. When ^{14}C- mannitol was used to estimate ECF volume this inhibition of the dilution of the marker was not observed (Table 4.5).

TABLE 4.1

THE EFFECT OF sSCT UPON PLASMA CALCIUM AND INORGANIC PHOSPHATE CONCENTRATION IN THE 19-DAY OLD CHICK EMBRYO

Calcium (mM)

Time	10 min	20 min	40 min	80 min
Control	2.635±0.050 (4)	2.508±0.033 (4)	2.610±0.020 (4)	2.603±0.025 (4)
500 ng	2.583±0.033 (4)	2.583±0.013 (5)	2.625±0.018 (5)	2.630±0.018 (4)
50 ng	2.613±0.023 (5)	2.583±0.035 (5)	2.620±0.013 (5)	2.590±0.030 (5)
5 ng	2.573±0.010 (4)	2.618±0.033 (4)	2.595±0.025 (4)	2.620±0.018 (4)

Phosphate (mM)

Time	10 min	20 min	40 min	80 min
Control	2.095±0.013 (4)	1.932±0.081 (4)	2.098±0.049 (4)	2.089±0.089 (4)
500 ng*	1.933±0.024 (4)	1.994±0.090 (4)	2.302±0.160 (5)	2.391±0.181 (4)
50 ng	2.163±0.174 (5)	2.036±0.077 (5)	2.164±0.102 (5)	2.202±0.086 (5)
5 ng	2.183±0.094 (4)	2.240±0.183 (4)	2.202±0.083 (4)	2.303±0.032 (4)

* ng of sSCT (4790 u/mg)

The numbers in each group are shown in parentheses

TABLE 4.2

THE EFFECT OF sSCT UPON PLAMSA VOLUME AND PLASMA MAGNESIUM CONCENTRATION IN 19-DAY OLD CHICK EMBRYOS

Plasma Volume (ml)

	Time			
Control	2.86±0.14 (4)	3.15±0.19 (4)	3.05±0.10 (4)	3.32±0.29 (5)
500 ng*	2.88±0.06 (4)	3.04±0.12 (4)	3.00±0.10 (4)	3.10±0.10 (4)
50 ng	2.69±0.14 (5)	3.05±0.10 (5)	2.94±0.17 (5)	3.31±0.22 (5)
5 ng	3.17±0.28 (4)	3.10±0.10 (4)	3.29±0.29 (4)	3.64±0.15 (4)

Magnesium (mM)

Time	10 min	20 min	40 min	80 min
Control	0.893±0.035 (4)	0.849±0.032 (4)	0.862±0.092 (4)	0.815±0.024 (4)
500 ng	0.906±0.056 (4)	0.894±0.088 (5)	0.900±0.018 (5)	0.883±0.091 (4)
50 ng	0.847±0.024 (5)	0.821±0.027 (5)	0.851±0.039 (5)	0.841±0.031 (5)
5 ng	0.809±0.088 (4)	0.817±0.036 (4)	0.812±0.032 (4)	0.893±0.049 (4)

* ng sSCT (4790 u/mg)

The numbers in each group are shown in parentheses.

TABLE 4.3

THE EFFECT OF sSCT UPON PLASMA SODIUM AND POTASSIUM CONCENTRATION IN 19-DAY OLD CHICK EMBRYOS

Sodium (mM)

Time	10 min		20 min		40 min	80 min	
Control	142.3±2.1	(12)	144.3±2.7	(12)	143.8±1.8 (12)	144.6±2.1	(13)
500 ng*	143.0±1.3	(4)	144.8±1.8	(4)	142.3±2.6 (4)	149.5±2.8	(4)
50 ng	179.8±4.6^{xxx}	(5)	185.8±1.1^{xxx}	(5)	160.8±9.4^{x} (5)	144.8±1.5	(5)
5 ng	146.6±4.3	(12)	141.8±2.3	(12)	139.5±2.1 (12)	139.8±2.1	(12)

Potassium (mM)

Time	10 min		20 min		40 min	80 min	
Control	6.93±0.22	(12)	7.31±0.21	(12)	6.91±0.18 (12)	6.33±0.19	(13)
500 ng	8.20±0.24^{xx}	(4)	8.00±0.14	(4)	7.23±0.23 (4)	6.95±0.26	(4)
50 ng	7.74±0.46	(5)	7.46±0.22	(5)	6.94±0.46 (5)	6.15±0.12	(5)
5 ng	6.45±0.23	(12)	7.11±0.18	(12)	6.53±0.24 (12)	6.61±0.17	(12)

* ng sSCT (4790 u/mg)

The numbers in each group are shown in parentheses, where n = 12-13 figures are derived from two separate experiments

x $P < 0.05$ xx $P < 0.01$ xxx $P < 0.001$

TABLE 4.4

THE EFFECT OF sSCT UPON ECF VOLUME, AS MEASURED BY THE DILUTION OF $^{32}SO_4$, IN 19 DAY OLD CHICK EMBRYOS

	ECF Volume (ml)			
	Time			
	10 min	20 min	40 min	80 min
Control	12.53±0.93 (8)	14.67±0.37 (7)	16.23±0.49 (8)	19.47±0.60 (8)
5 ng SCT	14.15±0.64 (8)	13.61±0.34 (8)	13.98±0.59 (8)	13.99±0.38 (8)
	NS	NS	$P < 0.02$	$P < 0.001$

NS - not significant

TABLE 4.5

THE EFFECT OF sSCT UPON ECF VOLUME, AS MEASURED BY THE DILUTION OF ^{14}C-MANNITOL, IN 19 DAY OLD CHICK EMBRYOS

	ECF Volume (ml)			
	Time			
	10 min	20 min	40 min	80 min
Control	15.43±1.42 (4)	16.06±0.91 (4)	19.58±0.49 (4)	19.30±0.95 (4)
5 ng CT	14.42±0.39 (4)	15.42±0.36 (4)	18.72±0.75 (4)	21.76±0.73 (4)
	NS	NS	NS	NS

NS - not significant

DISCUSSION

The lack of effect of CT upon plasma calcium, magnesium or inorganic phosphate is not unexpected and agrees with the work of Urist (1967) and Candlish and Taylor (1970). It must, however, be mentioned that the circulating levels of CT in 19-day embryos are already high compared to the concentrations in adult chicken and it may be that those high levels are already having a maximal effect (for instance, in opposing some hypercalcaemic stimulus). However, in experiment 2, we have shown that the plasma CT concentration can increase naturally to above 5 ng/ml, and on this basis we might have expected an increase from 2.9 to above 5 ng/ml to have some actions if CT is involved in the plasma homeostasis of calcium, inorganic phosphate or magnesium. In addition, although doses of 50 and 500 ng are admittedly unphysiological, one might have expected to observe some effect of these doses upon these values. The design of the experiment has not eliminated a possible rapid compensatory action of PTH which may explain the lack of effect of CT upon plasma calcium concentration.

Barlet (1972) Paillard et al (1972) and Bijvoet et al. (1972) have suggested possible renal actions of CT. These include an increase in the renal excretion of calcium, phosphate, sodium, potassium and water, under the influence of physiological levels of CT in sheep and man. No available publications have confirmed these effects in the chicken or other birds. The results obtained by the workers quoted above could not be explained by indirect action via PTH.

The excretion of an ion by the kidney is not always reflected by a fall in the concentration of that ion in the plasma. In fact most homeostatic mechanisms control the plasma concentration of a particular ion during periods when the intake, (in the diet) and the output, (via the kidney) is very variable. The failure to detect absolute changes in Ca, Mg, PO_4, Na or K does not therefore exclude a renal effect of CT, but merely indicates that this experiment was not designed to detect such changes. Obviously the renal output of the embryonic kidney would have to be monitored directly, but this would be very difficult to do.

One renal effect that should be possible to detect is a water diuresis. It might well be expected that the loss of water via the kidney would result in a reduction of ECF volume. When sulphate was used as an indicator of ECF volume this was in fact the apparent result.

The method depended upon the dilution of $^{35}SO_4$ by the ECF and the measured index was the CPM per unit volume of plasma. $^{35}SO_4$ equilibrates with the total ECF volume very rapidly (Polimen, 1974), and if a known total number of counts are injected into the ECF then the volume of the ECF can be determined (from the dpm/unit volume). This determination is complicated by the fact that sulphate is removed from ECF by the kidney and is taken up into cells. Thus, with increasing time, more and more $^{35}SO_4$ is removed from the ECF and hence the CPM per unit volume of plasma decreases and a calculation of the ECF volume based upon this dilution will result in an increasingly greater value for the ECF volume with time. If CT had acted to remove water from the ECF then this would have in part balanced the effect of the removal of $^{35}SO_4$, since the removal of both $^{35}SO_4$ and water, if occurring at the same rate would not alter the concentration of $^{35}SO_4$ in the plasma or ECF. This was observed at the dose level of 5 ng CT.

However, we decided to confirm this result using an alternate marker for measuring ECF volume (^{14}C-mannitol). Theoretically an ideal ECF marker should have the following properties:-

(1) Rapid and homogeneous distribution through the extra-cellular space and exclusion from the intra-cellular compartment.

(2) No metabolic transformation.

(3) Negligible osmotic effect.

(4) Elimination from the body should be measurable.

(5) No binding to plasma proteins or cell membranes.

(6) Negligible toxicity.

Of the commonly used ECF markers inulin diffuses slowly and is extracted rapidly; sulphate, thiosulphate, thiocyanate, sodium and bromine are all not confined to the ECF; sucrose and mannitol are metabolised and thiocyanate binds to plasma proteins.

Radioactive sulphate was chosen initially because it diffuses rapidly and is excreted slowly at low serum concentrations. It is however taken up by cells and metabolised but by comparing control and experimental values for ECF volume at each time interval this problem was overcome.

Similarly the results for ECF volume using ^{14}C-mannitol were compared with control values at the same time post-injection and table 4.5 clearly shows no effect of CT upon ECF volume. The conclusion must therefore be drawn that the CT had a direct effect upon sulphate, to

decrease its uptake by cells and/or reduce the rate of excretion of sulphate by the kidneys. In view of the ^{14}C-mannitol it would seem that there was no reduction of ECF volume under the influence of CT.

The changes in both sodium and potassium concentration occurred only at the higher CT doses, (50 and 500 ng respectively). Only one significant increase in plasma potassium was observed and does not merit further discussion. The increases in sodium at 10, 20 and 40 min, with a dose of 40 ng is interesting but difficult to explain. The failure of other doses of CT to have a similar effect does not support a suggestion of any important role for CT in sodium homeostasis. Indeed the available literature would suggest an opposite effect, if any, upon plasma sodium, via an increase in renal sodium clearance.

Experiment 7

The restrictive role of Calcitonin in chick embryos made hypercalcaemic by i.v. calcium injection.

The failure of CT to reduce normal levels of calcium in the chick embryo does not necessarily rule out a role for CT in calcium homeostasis. Copp et al., (1970) have shown that CT may be involved in the prevention of hypercalcaemia in the turkey, and Brown, Perey, Dent and Good (1968), suggested that the hypercalcaemic effect of PTH was prolonged in UBX birds compared to sham operated controls. Morphological changes indicating stimulation by hypercalcaemia of specific UB cells in chickens has been observed in laying hens by Urist (1967), in cockerels by Copp, Webber, Low, Kueh and Biely (1968), and in mature chicks by Chan, Cipera and Belanger (1968,1969). In addition, increasing the calcium content of the diet of young (3-4 week) chickens by addition of 4% $CaCO_3$ to the stock diet, resulted in raised plasma calcium concentrations and a definite hypertrophy and hyperplasia of the light, (CT secreting), cells of the UB gland.

The above evidence for a role for CT in calcium homeostasis in the chicken suggested that we might usefully pursue our investigations into the role of CT in the chick embryo by examining the effect of sSCT upon an artificially raised plasma calcium concentration.

EXPERIMENTAL

In order to eliminate the effect of circulating CT day 16 eggs were used in this experiment. At this age no CT is detectable in the plasma either by RIA or bioassay. Eggs were weight-matched in the range 59-64 g and divided randomly into four groups of five. Each group was injected intravenously with 0.1 ml of either buffer (0.1 m phosphate buffer, pH 7.35), buffer + 600 µg calcium (as $CaCl_2$), buffer + 50 ng sSCT (prepared just before use) or buffer + calcium + sSCT. The embryos were bled from the umbilical artery 15 min post-injection and the total plasma calcium determined by A.A.S.

RESULTS

The results and analysis of variance for this experiment are shown in table 4.6.

Compared to the controls, CT alone had no effect on the plasma calcium confirming the results of experiment 6. The injection of 600 µg of calcium resulted in an increase in plasma calcium concentration from 2.45 to 3.90 mM which was highly significant. In the presence of added calcium CT reduced the plasma calcium concentration from 3.90 to 3.47 mM, (15.6 to 13.9 mg/100 ml), suggesting that CT was able to reduce the degree of hypercalcaemia resulting from the addition of 600 µg of calcium to the embryos.

Although CT cannot be detected until day 17 it would appear that the mechanism by which CT is able to restrict hypercalcaemia is present in the 16-day embryo.

DISCUSSION

This experiment represents the first direct demonstration of the ability of CT to restrict hypercalcaemia in the chick embryo. Such an action may well be the role of CT in later embryos when the circulating levels reach very high values. Two questions, however, still remain. Firstly, by what mechanism does CT reduce plasma calcium concentration? Secondly, what are the factors producing a hypercalcaemic stimulus in chick embryos after day 17 of incubation?

At the present time we can only speculate as to the mechanism of action of CT in reducing hypercalcaemia. It is widely accepted that, in

TABLE 4.6

THE EFFECT OF CALCIUM AND CT UPON PLASMA CALCIUM CONCENTRATION (mM) IN THE 16-DAY CHICK EMBRYO

	- CT	+ CT	Mean
-Ca	2.45 ± 0.03 (5)	2.51 ± 0.05 (5)	2.48
+Ca	3.90 ± 0.18 (5)	3.47 ± 0.01 (5)	
Mean	3.18	2.99	3.68

Analysis of Variance

F ratios

Effect of Calcitonin	10.89	xx
Effect of Calcium	439.78	xxx
Interaction between CT and Calcium	18.00	xxx

mammalian systems, at least, the action of CT is to inhibit PTH induced bone resorption. (Wallach, Chausmer, Mittleman and Dimish, 1967; Hirsch and Munson, 1966). The combatting of experimentally induced hypercalcaemia in the rat has been explained by the action of CT upon bone resorption, whether the hypercalcaemia was induced by calcium (Talmage, Neuenschwander and Kraintz, 1965), PTH (Hirsch and Munson, 1966; Baghdiantz, Blanquet, Croizet, Moura and Tayeau, 1965; Gittes and Irvin, 1965) or by excessive vitamin D (De Luca, Morii and Melancon, 1968; Bugnon, Maurat, Lenys, Moreau and Rousselet, 1967).

The role of PTH in the chick embryo has not been determined but it has been suggested that the parathyroid glands are functional by day 17, (Venzke, 1947). It would seem that the embryos are capable of responding to exogenous PTH at an even earlier time since a dose of 10 USP units of PTH, injected into the air-sac of 15-day embryos resulted in a significant increase in plasma calcium concentration. One and two day old chicks have also been shown to respond to a similar dose of PTH (Lewis and Taylor, 1972) and in fact are very sensitive compared to adult birds. The fact that PTH is capable of modifying the plasma calcium in the embryo, together with additional evidence showing that the embryonic parathyroid gland has ultrastructural signs of differentiation, (Narbaitz, 1972; Stoeckel and Porte, 1970) and can respond in vitro to variations in the concentration of calcium in the medium (Narbaitz and Gartke, 1975; Feinblatt, Tai and Kenny, 1975) tend to indicate that the parathyroid gland may play an important role in the regulation of plasma calcium in the embryo, as in the adult bird.

Indirectly, the failure of sSCT to reduce a normal plasma calcium concentration, whilst it does restrict a hypercalcaemic stimulus, may also indicate that in the former situation PTH may be compensating for any fall in plasma calcium concentration below normal values. In general terms, therefore, one might argue that a hypercalcaemic stimulus induced by PTH results in the release of CT, which, by inhibiting PTH-induced bone resorption, results in the maintenance of a plasma calcium concentration slightly above the normal value. However, the cause of PTH release remains an unanswered question.

Experiment 8

The effect of sSCT upon plasma ^{45}Ca and $^{32}PO_4$.

Radioisotopes of calcium have been employed by a number of investigators to study the mechanism of action of CT and PTH. If bone is deep labelled with ^{45}Ca two to three weeks before PTH administration the effect of the PTH will be to stimulate resorption of the labelled bone and thus increase the amount of ^{45}Ca in the plasma. Alternatively, given that a constant amount of PTH in the circulation is maintaining a certain level of plasma ^{45}Ca , then the effect of CT administration, by inhibiting PTH-induced bone resorption, will be to decrease the amount of circulating ^{45}Ca, together with a decrease in total calcium concentration. Short term studies in which ^{45}Ca and CT were administered together in the rat (Johnston and Deiss, 1966) resulted in no change in the rate of disappearance of the radioisotope, but a fall in total calcium concentration and hence an increase in the specific activity of the plasma ^{45}Ca. Long term studies by the same authors demonstrated that CT had no effect upon the specific activity of the plasma. O'Riordan and Aurbach (1968) administered CT 3 h after ^{45}Ca injection, and found essentially similar effects as the short term study of Johnston and Deiss, i.e. no effect upon the disappearance of ^{45}Ca from the plasma but the normal decline in specific activity was interrupted. These results are in agreement with a mechanism of action of CT involving the inhibition of PTH induced bone resorption, since PTH acts upon well established bone.

Whilst most studies regarding the actions of CT have been concerned only with the effects of the hormone on calcium and its radioisotopes some investigators have particularly emphasised a relationship between CT and phosphate metabolism. As well as its hypocalcaemic action in mammalian species, a hypophosphataemic action is also well established. Hirsch (1968) reported that the hypocalcaemic action of CT could be enhanced by the simultaneous administration of phosphate, and Kennedy and Talmage (1971) have in fact postulated that the hypocalcaemic action of CT might well involve an initial early effect upon phosphate metabolism.

Talmage, Anderson and Cooper, (1972) noted that the effects of CT upon $^{32}PO_4$ were in marked contrast to those upon ^{45}Ca. In both short (5 min) and long term (2 week) labelling experiments the effect of CT resulted in the loss of both $^{32}PO_4$ and phosphate from the plasma, without

changing the specific activity. These results were interpreted as a rapid increase in the movement of phosphate out of the plasma as a result of the action of CT. Whether CT acts separately upon these two ions (PO_4 and Ca) or whether its effect upon PO_4 produces a subsequent effect on calcium is questionable, but in later work (Talmage, Matthews, Martin, Kennedy, Davis, Roycroft, 1975) the latter effect was emphasised. These authors tentatively suggested that the hypocalcaemia produced by CT is secondary to the formation of a calcium phosphate complex in and around osteocytes and lining cells in the bone. They suggested that this complex, which is normally prevented from transforming to apatite crystals by the presence of an inhibitor, reduces the availability of calcium for rapid transport to the ECF. The reduction in calcium flux from bone to ECF results in a rapid and transient hypocalcaemia.

Similar experiments utilising radioisotopes of calcium and phosphate have not been done in the chicken, and we therefore examined the effect of sSCT upon plasma ^{45}Ca and $^{32}PO_4$ in chick embryos.

EXPERIMENTAL

Randomly selected embryos at days 16-19 were injected I.V. with either a control solution (0.1 m acetate buffer pH 7.35) containing 1 µCi ^{45}Ca and 1 µCi $^{32}PO_4$ or the control solution plus 10 ng sSCT. Embryos were bled at 5, 10, 15, 30, 60 or 120 min post-injection and duplicate 0.1 ml plasma samples were counted in a Beckman liquid scintillation counter with suitable channel settings to distinguish between the two isotopes. The amount of each isotope in a 0.1 ml sample in experimental embryos (+ CT) was compared to the control values (- CT) at the same time interval post-injection, and expressed as a percentage change from the control. Positive values indicate a retention of isotope and negative values indicate a loss of isotope, compared with the controls.

0.1 ml plasma samples were also taken for total calcium and inorganic phosphate determinations.

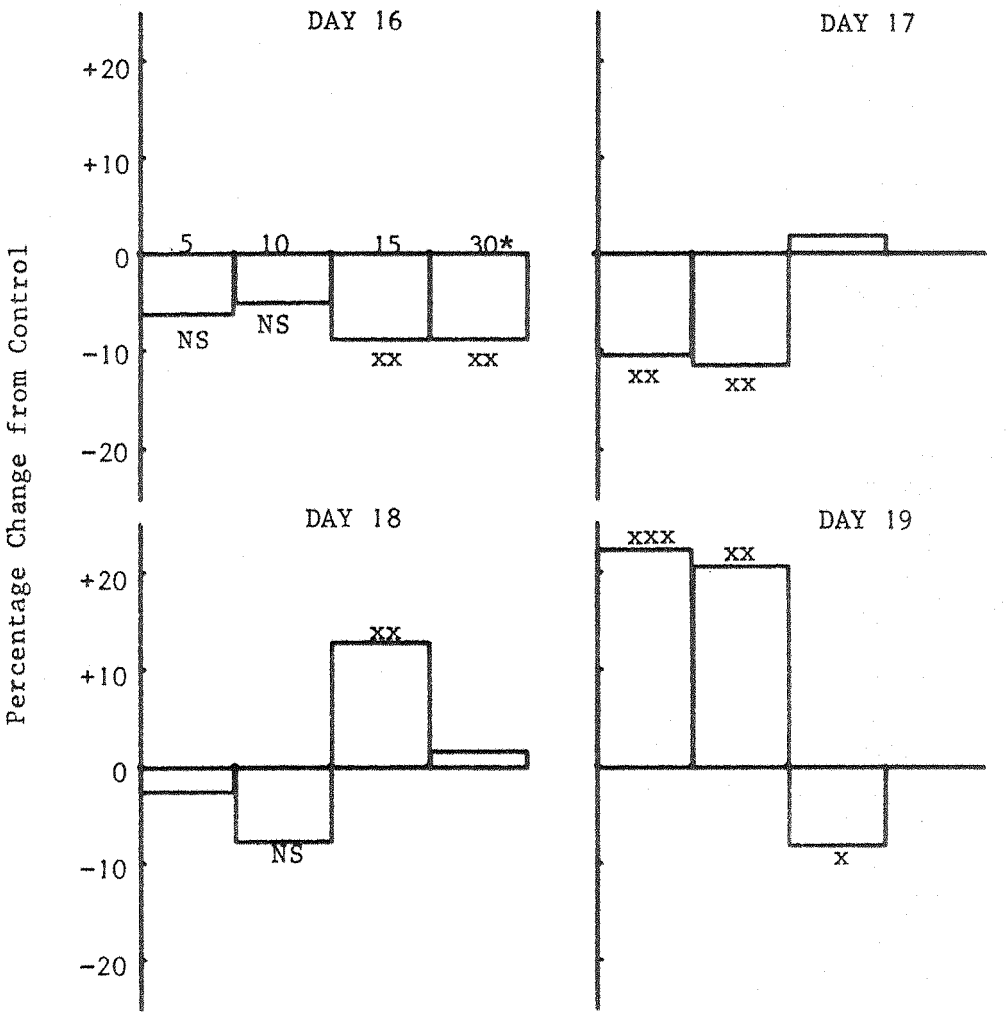
RESULTS

The results are shown in Figs 4-1, 4-2, 4-3 and 4-4.

Fig. 4-1 and Fig. 4-2.

(1) At all times post-injection on day 16 there was an increased

FIG. 4.1 The effect of sSCT upon the loss of ^{45}Ca from the plasma of chick embryos



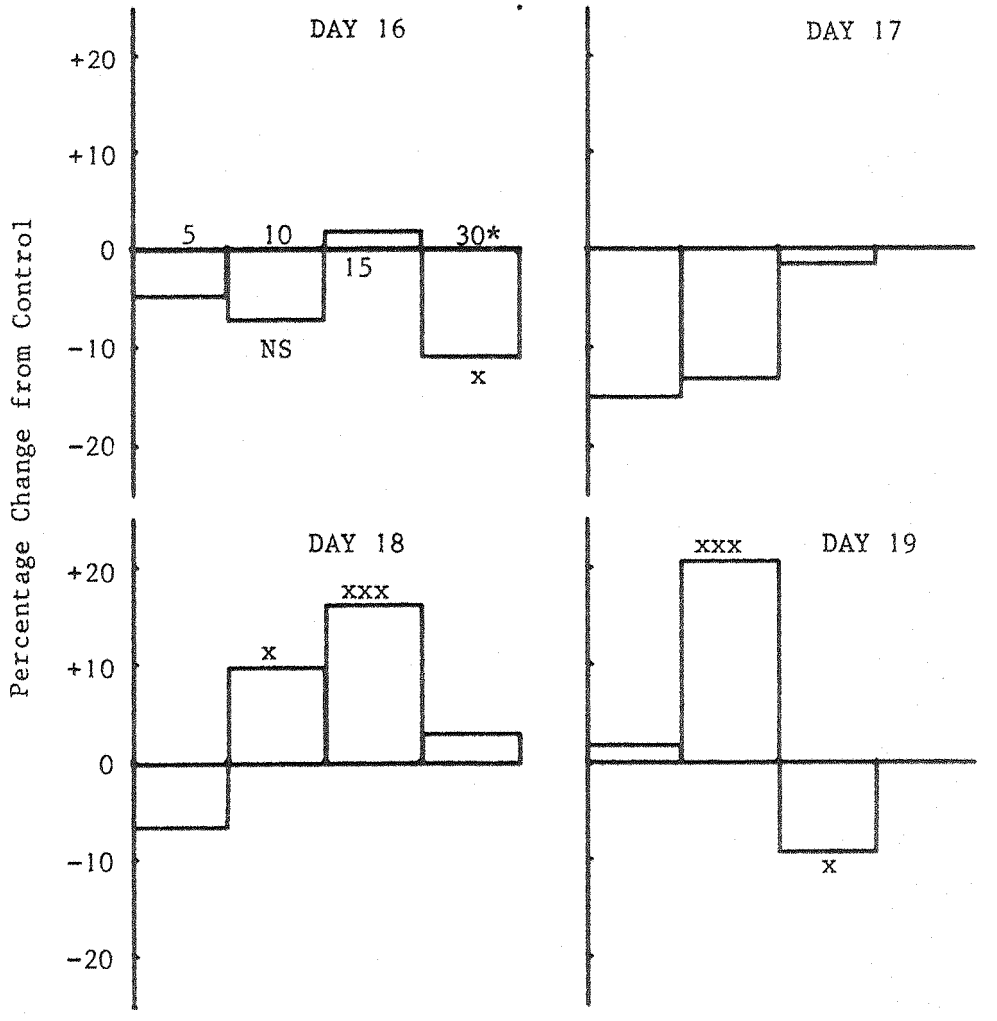
* X-axes in all cases are 5, 10, 15 and 30 min post ^{45}Ca/CT injection

n = 6-8 for day 16, 17 and 18 and 10-11 for day 19

x $P < 0.05$ xx $P < 0.01$ xxx $P < 0.001$

NS not significant

FIG. 4.2 The effects of sSCT upon the loss of $^{32}PO_4$ from the plasma of chick embryos



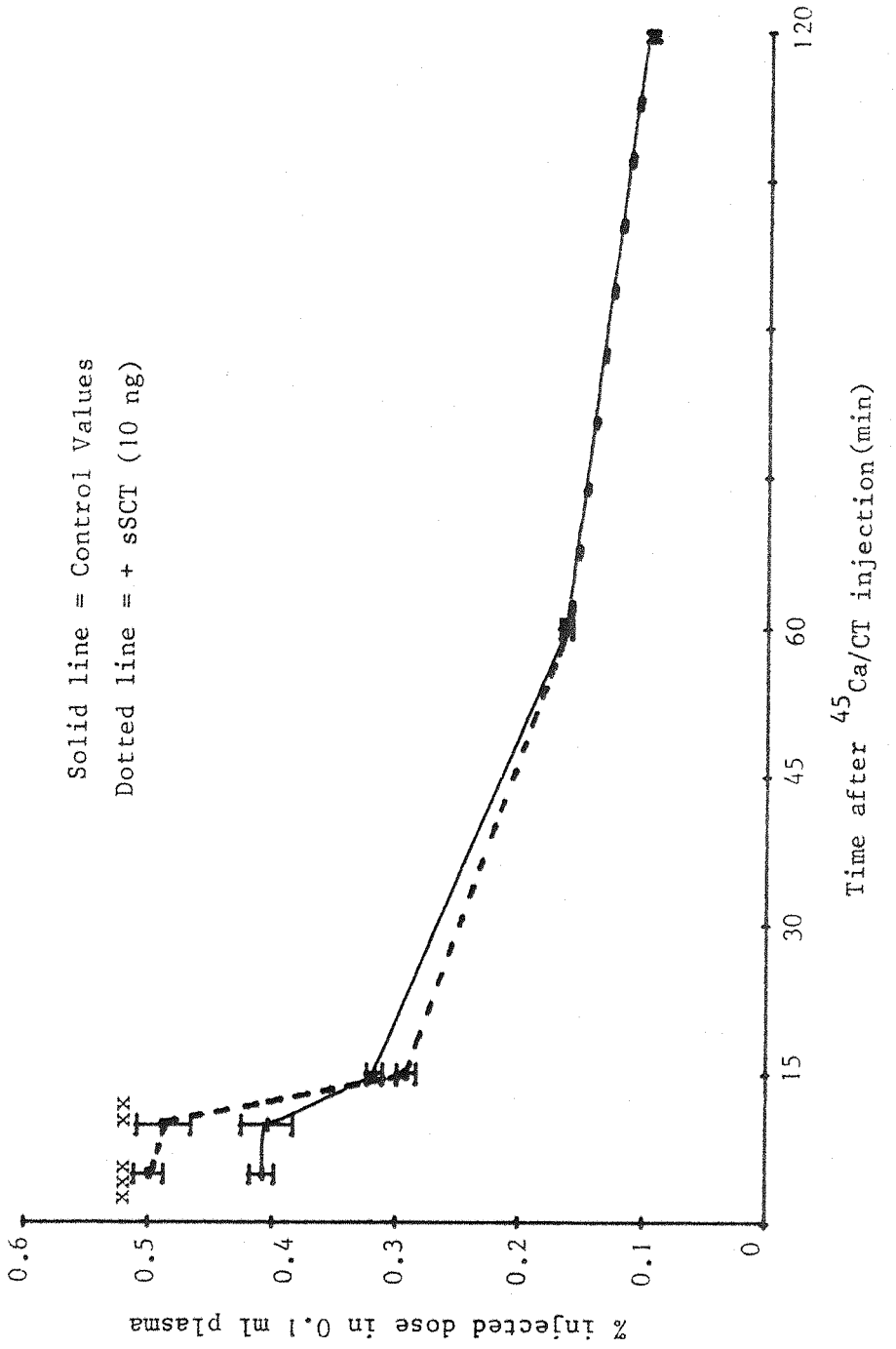
* x-axes in all cases are 5, 10, 15 and 30 mins post $^{32}PO_4$/CT injection

n = 6-8 for days 16, 17 and 18 and 10-11 for day 19

x $P < 0.05$ xx $P < 0.01$ xxx $P < 0.001$

NS not significant

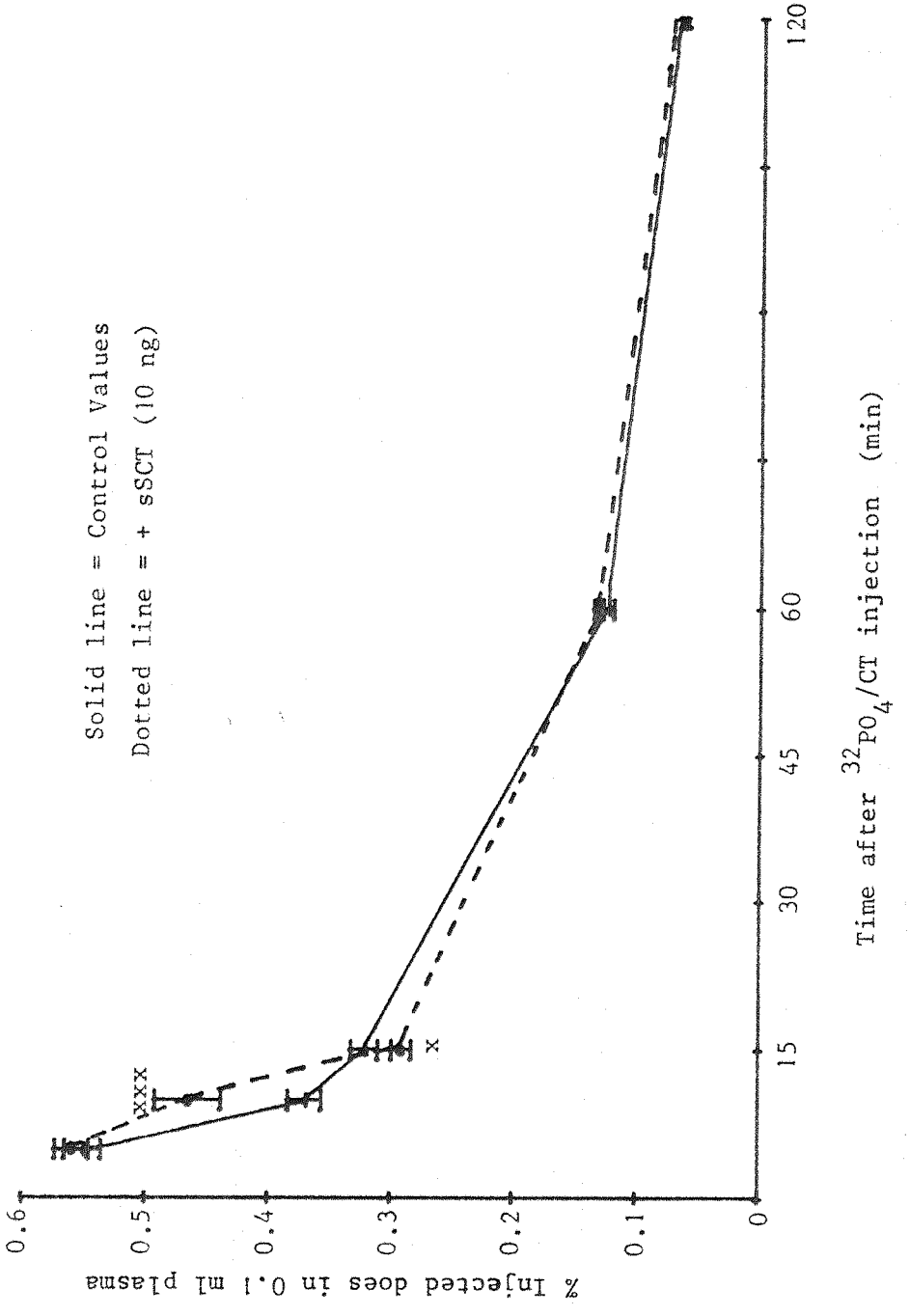
FIG. 4.3 The effect of sSCT upon the loss of ^{45}Ca from the plasma of day 19 chick embryos



xx P < 0.01 xxx P < 0.001

n = 10–11 for 5, 10 and 15 min and 6–7 for 60 and 120 min.

FIG. 4.4 The effect of sSCT upon the loss of $^{32}PO_4$ from the plasma of day 19 chick embryos



x $P < 0.05$ xxx $P < 0.001$

n = 10-11 for 5, 10 and 15 and 6-7 for 60 and 120 min.

loss of ^{45}Ca from the plasma compared to the control values which was significant at 15 and 30 min. There was also an increased loss of $^{32}PO_4$ (with the exception of a small, non-significant retention at 15 min) which was significantly different from controls at 30 min.

(2) In day 17 embryos there was a significant loss of both ^{45}Ca and $^{32}PO_4$ at 5 and 10 min post-injection, with no significant difference at 15 min.

(3) In day 18 embryos there was a significant retention of ^{45}Ca at 15 min and a significant retention of $^{32}PO_4$ at 10 and 15 min.

Fig. 4-1 with Fig. 4-3.

In 19 day embryos there was a transient, but significant retention of ^{45}Ca after 5 and 10 min with a small and just significant loss of ^{45}Ca at 15 min. Thereafter values for control and + CT group were identical.

Fig. 4-2 with Fig. 4-4.

In 19 day embryos there was a significant retention of $^{32}PO_4$ at 10 min and a significant loss of $^{32}PO_4$ at 15 min and thereafter values for control and CT groups were identical.

As expected from Experiment 6 CT produced no detectable changes from control values in total calcium or inorganic phosphate concentrations. No figures for specific activity (s.a) are presented therefore, but a loss of isotope must have decreased the s.a. whilst the retention of isotope must have increased the s.a. It was also noted that the ratio of $^{45}Ca/^{32}PO_4$ in the plasma invariably increased with the duration of the experiment at each of the ages of embryo investigated and that in all groups injected with CT the $^{45}Ca/^{32}PO_4$ was slightly, but not always significantly, higher. This suggests that phosphate is removed more rapidly from the plasma than calcium and that CT enhanced this difference in the rate of removal.

DISCUSSION

The variability with age in the effect of sSCT upon plasma ^{45}Ca and $^{32}PO_4$ of the embryo was unexpected. Generally, in days 16 and 17 sSCT at a dose of 10 ng resulted in a loss of both ^{45}Ca and $^{32}PO_4$, whilst in day 18 and 19 this effect was reversed and a significant retention of both isotopes was observed. Whether the effect of CT was a retention or

a loss of isotope it was transient in all experiments. This is clearly shown in Figs 4-3 and 4-4. No significant changes from control values were noted in any experiment at post-injection times greater than 15 min.

Talmage, Anderson and Cooper (1972) noted a significant reduction in total plasma phosphate and $^{32}PO_4$ 20 min post-injection of CT and this difference increased to a maximum 1 h post-injection. No change in the s.a. of plasma $^{32}PO_4$ was observed and the results were essentially similar whether the isotope was injected simultaneously with, or 2 weeks prior to, the hormone. The same authors found that the effect of CT upon plasma calcium and ^{45}Ca was to decrease total calcium and to increase the s.a. of ^{45}Ca in both long and short term experiments. The absolute plasma ^{45}Ca content was marginally reduced if the ^{45}Ca was administered simultaneously but was very significantly reduced in long term labelled rats.

In days 18 and 19 a significant retention of ^{45}Ca, without any concurrent change in total plasma calcium concentration, is equivalent to an increase in s.a. of ^{45}Ca which is in agreement with the effect of CT upon plasma ^{45}Ca s.a. reported in the rat by Talmage et al (1972) O'Riordan et al (1968) and Johnston et al (1966). The effect in the chick embryo, however, was much more rapid. The retention of $^{32}PO_4$ (and therefore an increase in s.a. of plasma $^{32}PO_4$) which resulted from the action of CT in day 18 and 19 of incubation is not in agreement with the work of Talmage et al (1972) and there is certainly no increase in the rate of removal of $^{32}PO_4$ from the plasma.

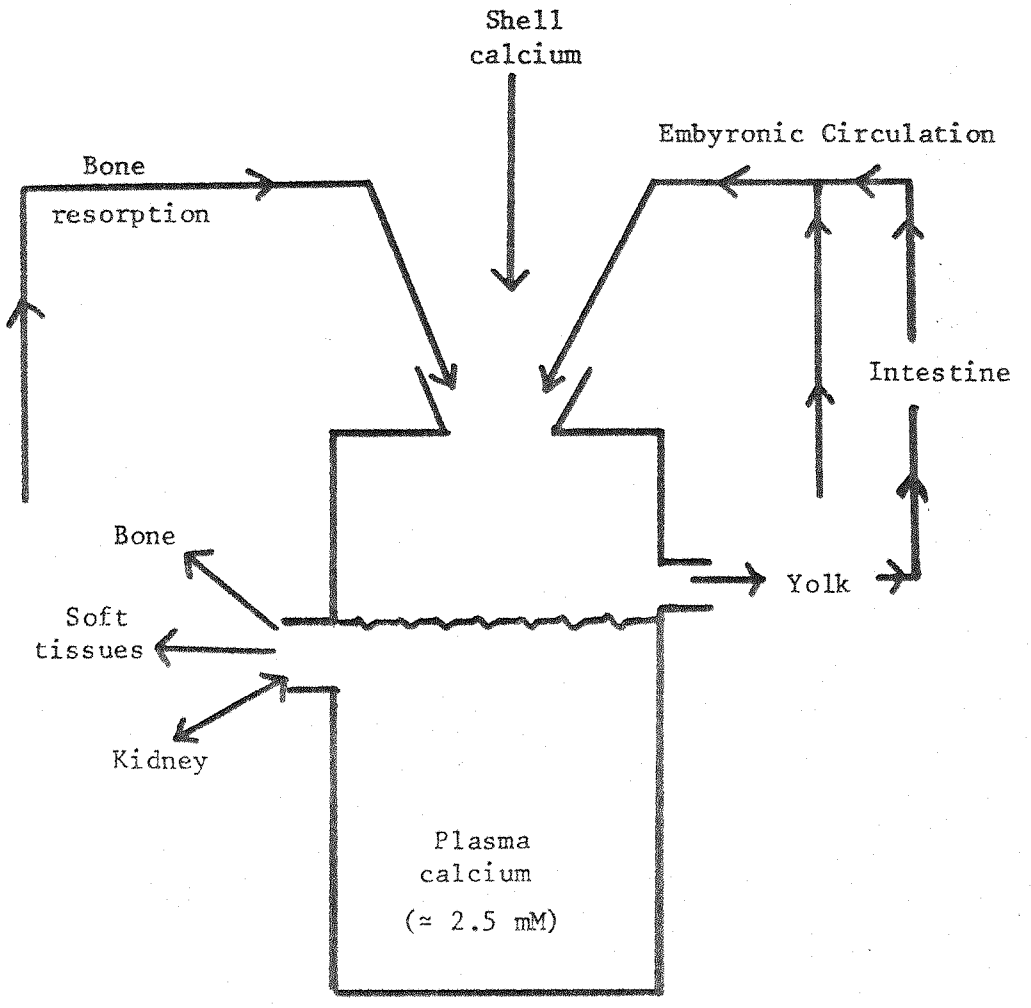
The transient nature of the response of 18 and 19 day chick embryos to sSCT may be explained as an early effect of the hormone. In a series of papers (Caniggia, Gennari, Bencini and Borello, 1969; Caniggia, Gennari, Piantelli and Vattimo 1972; and Caniggia and Gennari 1975) some interesting early effects of CT upon plasma calcium and ^{45}Ca in human patients have been shown. In terms of total plasma calcium, the effect of an I.V. dose of 135 MRC units of sSCT was a transient increase from 5-15 min prior to the expected hypocalcaemic action. The same dose of sSCT also resulted in the transient retention of ^{47}Ca. The same authors did not examine the effect of sSCT upon plasma phosphate or $^{32}PO_4$, but explained the effect of sSCT upon calcium and ^{45}Ca in terms of an immediate shift of calcium and ^{45}Ca from reservoirs with high specific activity to the circulation and thereafter in the urine, and a more persistent direct effect on the kidney. They further suggested

that the reservoirs with a high specific activity were in fact bone cells, but their patients were injected with the ^{47}Ca 2-8 h before hormone administration and not simultaneously. In order to explain the retention of ^{45}Ca in 18- and 19-day embryos in a similar manner we would have to suggest that ^{45}Ca is very rapidly taken up by bone cells and then released again, or that CT prevents the entry of ^{45}Ca into the cells. Presumably, a loss of calcium, possibly via the kidney, must also occur in order to prevent an increase in total plasma calcium concentration.

If we attempt to explain the retention of ^{45}Ca and $^{32}PO_4$ within chick embryo plasma without a change in total calcium or phosphate in terms of a calcium and phosphate homeostatic mechanism, rather than a transient, short term effect, unrelated to the metabolism of either calcium or phsophate, then it would appear that only two explanations are possible. Fig. 4-5 may be of help in these explanations.

To maintain a constant plasma total calcium and inorganic phosphate the entry of these ions (from the shell, yolk, intestine or via bone resorption) must be balanced by their loss (to the bone, soft tissues, or yolk). In the normal state there is a net movement of calcium and phsophate to the bone, and to a small extent to the soft tissues, whilst calcium can still be deposited in the yolk in 18 and 19 day embryos. If CT acts by inhibiting bone resorption then the calcium and phosphate entering the plasma from other sources must either be excreted by the kidney or deposited into the yolk-sac or we might suggest that CT also inhibits the entry of calcium and phosphate from the chorio-allantoic membrane, yolk and intestine.

The latter possibility, in effect, is a reduction in the net flux of calcium and phosphate through the embryonic circulation. It must be emphasised that an effect of CT upon bone resorption without an effect on the kidney (or possibly the yolk-sac) or a concomitant effect upon the entry of calcium and phosphate would result in changes in total calcium and inorganic phosphate concentrations which are not observed experimentally. Some evidence is available to suggest that CT can act upon the intestine, to inhibit phosphate absorption (Tanzer and Navia 1973), but whether such an action could be invoked to reduce phosphate transport in the embryonic intestine or yolk-sac membrane is speculative. Terepka et al (1971) found no effects of CT or PTH upon the calcium transport of chick embryo chorio-allantoic membranes <u>in vitro</u>. It would



Sources of Calcium

(1) Shell
(2) Yolk
(3) Intestine
(4) Bone Resorption
(5) Renal Tubular Reabsorption

Loss/Deposition of Bone

(1) Bone
(2) Yolk
(3) Renal Excretion
(4) Soft Tissues

FIG. 4.5 Diagrammatic representation of calcium homeostasis in the chick embryo

seem that an action upon both bone and kidney is the more likely explanation of the experimental results.

The discussion is further complicated by the opposite effect of CT upon ^{45}Ca and $^{32}PO_4$ in 16 and 17 day embryos. The previous experiment demonstrated the ability of sSCT to restrict experimentally induced hypercalcaemia, presumably by inhibiting the entry of additional calcium into the system, and most probably by inhibiting PTH induced bone resorption. At normal calcium concentrations this effect is not observed, the explanation for which may be a rapid compensatory action of PTH. Since the total amount of ^{45}Ca injected into embryos was very small, and did not artificially create a hypercalcaemia, it may be that the effects upon ^{45}Ca and $^{32}PO_4$ are due, not to CT, but to a CT induced increase in circulating PTH. However, this explanation could equally apply to 18 and 19 day embryos, unless such an effect of CT upon PTH secretion was not possible until day 18. This seems most unlikely.

Regardless of the age of the embryo, the transient nature of the response might suggest that the effects of sSCT upon plasma ^{45}Ca and $^{45}PO_4$ are short-term and unrelated to calcium and phosphate metabolism. CT has been shown to stimulate the uptake of ^{45}Ca by a variety of cells (Borle, 1969 and 1973) not all associated functionally with calcium homeostasis, and to decrease efflux of calcium from kidney cells (Borle, 1969). It is, however, again difficult to explain the difference in the effect of CT with increasing age of development of the chick embryo.

One remaining possibility, which might explain the age dependent effect of CT, is that the total circulating concentration of CT in 18 and 19 day embryos will be greater than in 16 and 17 day embryos since high circulatory amounts of CT were already present prior to the injection of an additional 10 ng (Experiment 2). It might be that, in 16 and 17 day embryos, we are in fact observing the true effect of sSCT upon plasma ^{45}Ca and $^{32}PO_4$, whilst in 18 and 19 day embryos, the effect is complicated, possibly by direct CT stimulation of PTH release. This latter effect of CT has been shown by Dufresne and Gitelman (1972) and Fischer, Oldham, Sizemore and Arnaud (1971); and Fischer, Blum and Binswanger (1973), but Care, Bates, Swaminathan, Scanes, Peacock, Mawer, Taylor, De Luca, Tomlinson and O'Riordan (1975) using an *in vivo* parathyroid perfusion system with high, (23-660 mU/ml) concentration of sSCT, could only find a stimulation of PTH secretion in one out of four

experiments. It must, however, be mentioned that such an effect of CT upon PTH secretion rate may be pharmacological rather than physiological since the amount of CT required to produce such an effect was always high.

CHAPTER 5

The experiments to be reported in this chapter were designed to investigate some possible stimuli for the release of CT in the chick embryo and to observe the effect of the removal of CT from the circulation of the embryo. In addition, the location of a CT peak specifically at the pipping stage (Experiment 2) was investigated by artificially advancing or retarding the onset of pipping. The results of these experiments will be discussed collectively at the end of the chapter.

Experiment 9

Changes in plasma Calcitonin resulting from a calcium challenge in the chick embryo.

Calcium has been shown to stimulate the release of CT from C-cells both in mammalian thyroid glands and in sub-mammalian ultimobranchial glands. Specifically the UB glands of the chicken have been shown to respond to a calcium challenge in the perfused isolated UB gland of a hen (Ziegler, Telib and Pfeiffer, 1969), in a calcium infused anaesthetised cockerel (Copp, Brooks, Low, Newsome, O'Dor, Parkes, Walker and Watts, 1970) and in an in vitro UB gland culture system, (Feinblatt, Raisz and Kenny, 1973; Nieto, L-Fando and R-Candela, 1975). Whether or not the chick embryo UB gland is capable of responding in vivo to a calcium challenge was not known, and was therefore the subject of this experiment.

EXPERIMENTAL

Fertile chick embryos at 17 days of incubation were randomly removed from an incubator and injected, intravenously with 0.1 ml physiological saline (9 g/l NaCl) containing 0, (control embryos), 200, 400 or 800 µg of calcium (as $CaCl_2$). Embryos were bled by cardiac puncture 10 min following the injection and duplicate 0.1 ml plasma samples were used for the determination of total plasma calcium by A.A.S; the remaining plasma was stored under liquid nitrogen until assayed for CT by RIA. Individual plasma samples were assayed for CT undiluted and diluted 1:1 with distilled water; the average concentration, expressed as ng/ml sSCT equivalents, was then calculated. Groups were compared for statistical significance by Students t-test.

RESULTS

The results are shown in table 5.1.

A highly significant hypercalcaemia was produced by the injection of all three doses of calcium compared to the saline injected controls. The degree of hypercalcaemia approximated to calculated values based upon an ECF volume of around 10 ml (3.2, 3.5 and 4.6 mM for the 200, 400 and 800 µg injections, respectively).

All three doses of calcium resulted in a significant increase in the circulation plasma CT concentration. From a normal level of 1.53 ng/ml the plasma CT rose to 2.51, 3.66 and 3.73 ng/ml following the injection of 200, 400 and 800 µg calcium respectively.

Experiment 10

The involvement of the β-adrenergic system in the stimulation of Calcitonin release from the UB gland of the chick embryo.

In comparing the histological structure of ultimobranchial and thyroid C-cells, Hodges (1970) noted the relative abundance of nerve terminals in the UB gland of the chicken compared to the rat. The possibility of a partial control of UB function via a neural pathway in the chicken and other lower vertebrates, has been suggested, (Hodges and Gould, 1969; Gould and Hodges, 1971; Care and Bates, 1973; Ziegler et al, 1970). It has been further suggested (Bates, Phillipo and Lawrence, 1970; Care, Bates and Citelman, 1971) that such a neural pathway may involve the β-adrenergic system, since the β-adrenergic agent isoprenaline (isoproterenol) stimulates and the β-blocker, propranolol, inhibits the release of CT from C-cells. We wished to investigate the response of the chick embryo UB gland to these two agents and in particular we were interested to observe the effect of the β-blocking agent, propranolol, since a fall in the circulating levels of CT in the embryo, as a result of the action of this drug, would indicate that the release of CT in the late chick embryo might involve a neural mechanism.

EXPERIMENTAL

Fertile chick embryos were removed from an incubator at day 19 and randomly divided into groups of 10. Doses of 10, 1, 0.1, 0.01 or 0 mg DL-propranolol HCl were injected onto the air-sac membrane and the

TABLE 5.1

THE EFFECT OF INJECTIONS OF VARYING AMOUNTS OF CALCIUM OR THE CONCENTRATION OF TOTAL CALCIUM AND CALCITONIN IN THE PLASMA OF CHICK EMBRYOS AFTER 10 MIN.

	CONTROL	200 µg Ca	400 µg Ca	800 µg cA
Plasma Calcium (mM)	2.58 ± 0.05	3.20 ± 0.04	3.51 ± 0.09	4.57 ± 0.17
Plasma CT (ng/ml sSCT equivalents)	1.53 ± 0.07	2.51 ± 0.24 xx	3.66 ± 0.53 xx	3.73 ± 0.50 xxx
n	8	8	8	8

xx $P < 0.01$

xxx $P < 0.001$

injected volume = 0.1 ml

embryos were then bled by cardiac puncture 2 h later.

Physiological saline (9 g/l NaCl) was used as the control and diluent for all experiments involving propranolol,but the stability of isoprenaline required the drug to be diluted with 10^{-4} M ascorbic acid, pH 3.0, and the control embryos were also injected with this solution. In all cases duplicate 0.05 ml plasma samples were taken for total calcium determination; 0.2 ml plasma was diluted with 0.2 ml distilled water and the total inorganic phosphate determined by use of an auto-analyser and any remaining plasma was stored under liquid nitrogen for CT determination by RIA.

In a further experiment, 1 mg propranolol was injected into two groups of 10 eggs, but plasma samples were taken at 30 min and 1 h post-injection. Calcium, inorganic phosphate and CT were determined as above.

RESULTS

Comparing the control groups for the propranolol (saline) and isoprenaline (ascorbic acid) experiments, the plasma calcium concentrations were very similar, (2.56 mM and 2.57 mM respectively), but in the isoprenaline experiment the CT concentrations were undetectable (< 0.42 ng/ml sSCT equivalents) whereas the saline injected propranolol controls had a plasma CT concentration of 2.2 ng/ml, which is close to the value found in experiment 2 for 19 day embryo plasma.

There were no significant changes in plasma inorganic phosphate concentration with the exception of a small fall resulting from the injection of 1 mg isoprenaline 2 h post-injection.

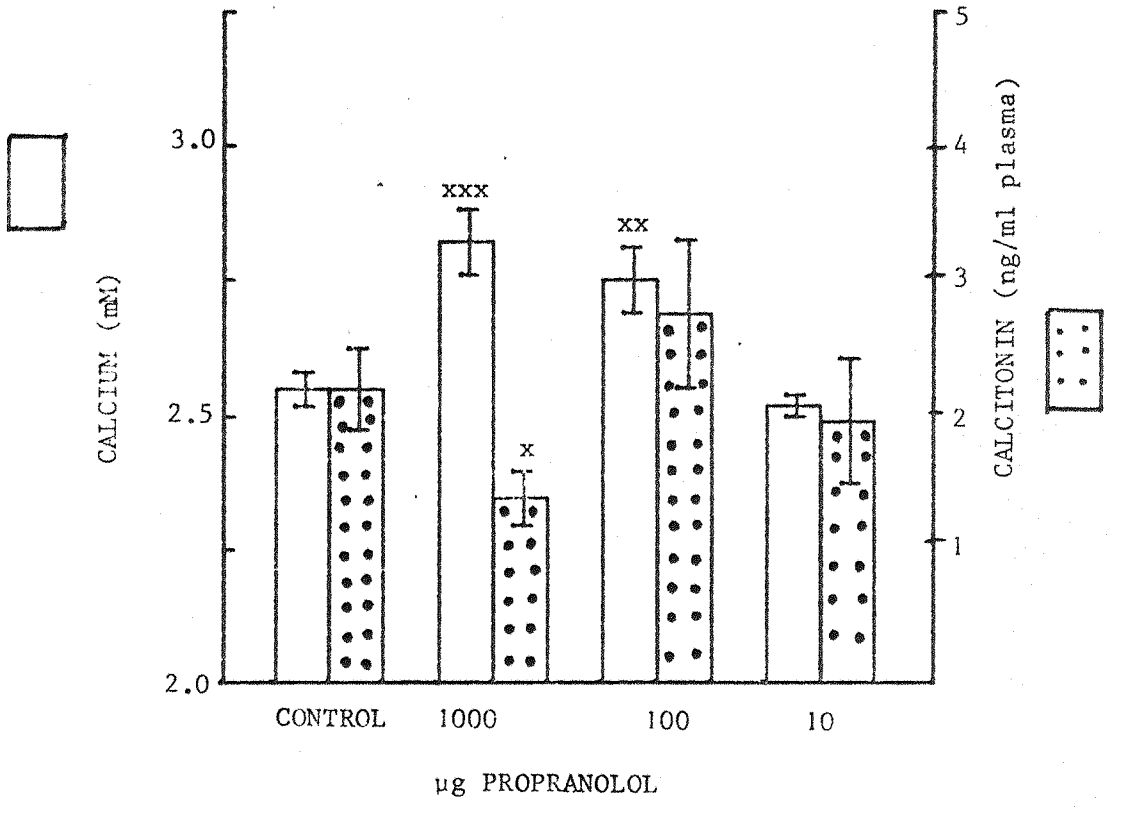
The results are shown in Fig. 5-1, 5-2 and 5-3.

Propranolol

(1) The 10 mg dose of propranolol was lethal in all 10 embryos injected. This served to demonstrate that an injection of the drug onto the air-sac membrane was a suitable route for the administration of propranolol.

(2) Plasma calcium was significantly raised from the control value of 2.56 mM to 2.82 and 2.75 mM by doses of 1 and 0.1 mg respectively. The lowest dose of 0.01 mg had no significant effect upon plasma calcium.

FIG. 5.1 The effect of PROPRANOLOL upon plasma CALCIUM and CALCITONIN in 19 day old chick embryos 2 h post-injection



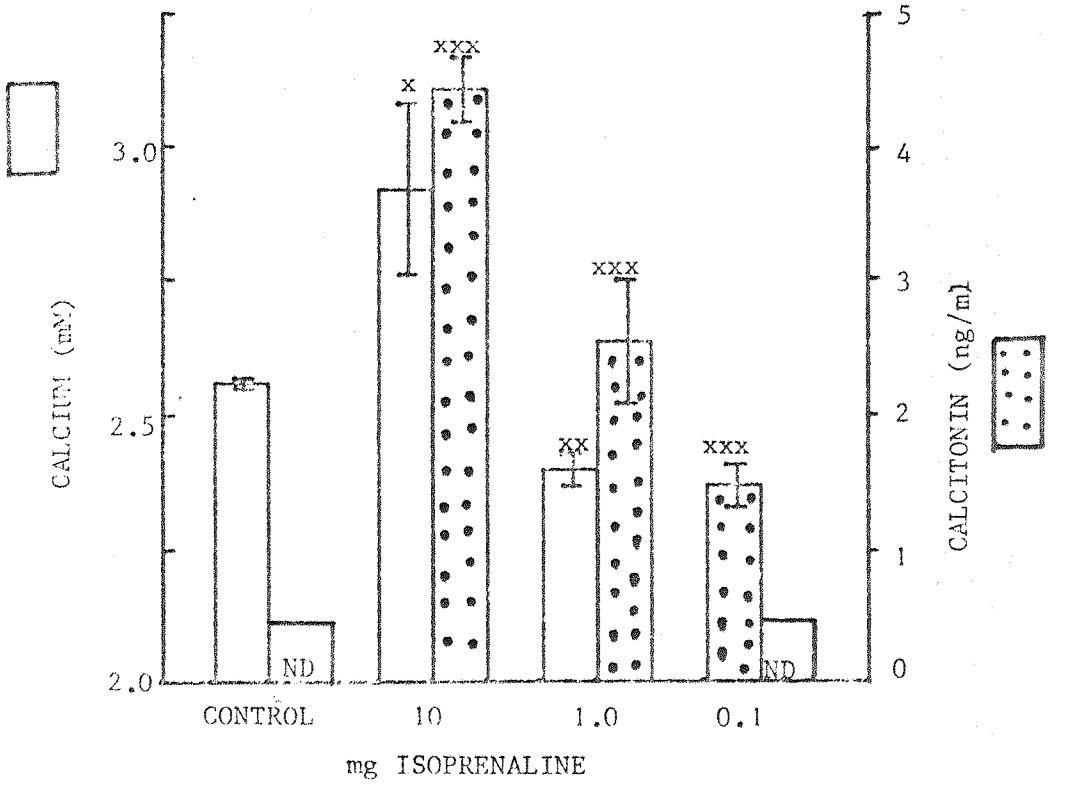
n = 10 for all groups

The effect of propranolol on plasma inorganic phosphate

Dose of Propranolol (mg)	Plasma Inorganic Phosphate (mM)		N
CONTROL (0)	1.50 ± 0.05	NS	10
0.01	1.60 ± 0.08	NS	10
0.1	1.70 ± 0.09	NS	10
1.0	1.53 ± 0.06	NS	10

x P < 0.05
xx P < 0.01 NS Not significant
xxx P < 0.001

FIG. 5.2 The effect of ISOPRENALINE upon plasma CALCIUM and CALCITONIN in 19 day old chick embryos 2h post-injection



ND - Not detectable (< 0.4 ng/ml)

Control injected with 10^{-4} ascorbic acid, pH 3.0

n = 10 for all groups

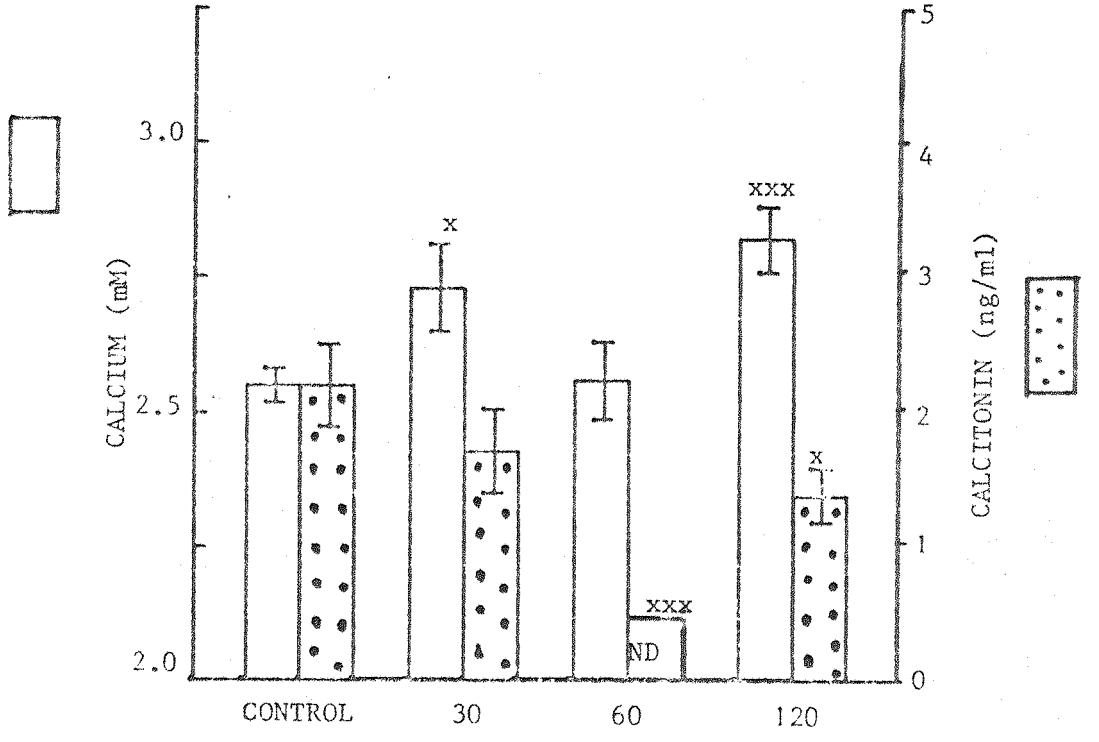
The effect of isoprenaline upon plasma inorganic Phosphate

Dose of Isopreanline (mg)	Plasma Inorganic Phosphate (mM)		N
CONTROL (0)	1.53 ± 0.09		10
0.1	1.34 ± 0.04	NS	10
1.0	1.19 ± 0.05	xx	10
10	1.54 ± 0.11	NS	10

x P < 0.05 xxx P < 0.001

xx P < 0.01 NS Not significant

FIG. 5.3 Time course for the action of 1 mg PROPRANOLOL upon plasma CALCIUM and CALCITONIN in 19 day old chick embryos



Time after injection (min)

n = 10 for all groups

ND = not detectable (< 0.4 ng/ml)

Time course showing the effect of 1 mg Propranolol upon Plasma Inorganic Phosphate

Time after Injection (min)	Plasma Inorganic Phosphate		N
30	1.39 ± 0.07	NS	10
60	1.48 ± 0.06	NS	10
120	1.53 ± 0.06	NS	10
CONTROLS	1.50 ± 0.05	NS	10

x $P < 0.05$ xxx $P < 0.001$

xx $P < 0.01$ NS Not significant

(3) The plasma CT concentration significantly fell from the control value of 2.20 ng to 1.39 ng following the injection of 1 mg propranolol, but lower doses were without effect.

(4) When samples were taken at 30, 60 and 120 min following the injection of 1 mg propranolol it was observed that the plasma CT concentration fell to undetectable levels by 60 min but at 120 min CT was again detectable, (1.39 ng/ml), but still significantly lower than the control value.

(5) In the same time course experiment plasma calcium concentration was significantly increased at 30 and 120 mins, but was not significantly different from the control value at 60 min following the injection of 1 mg propranolol.

Isoprenaline

(1) The highest dose of 10 mg isoprenaline resulted in a significant increase in plasma calcium and a highly significant increase in plasma CT.

(2) The intermediate dose of 1 mg isoprenaline also resulted in a highly significant increase in plasma CT but the total plasma calcium concentration fell significantly below the control value.

(3) The lowest dose of 0.1 mg isoprenaline did not alter the plasma CT concentration, (which was undetectable), but the plasma calcium remained significantly lower than the control value.

Experiment 11

The effect of the removal of circulating CT upon plasma calcium concentration in the chick embryo.

If the concept of CT acting as a restricting agent in the control of plasma calcium in the chick embryo is correct then an important question is to determine the effect of the removal of circulating CT. In the adult bird, or even a newly-hatched chick, this would be possible by direct surgical removal of the UB glands, but such surgery in the in ovo embryo would be very difficult to attain whilst maintaining a viable embryo. An alternative method was therefore chosen, namely to inject sufficient of a CT-specific antibody which would bind the circulating CT and prevent its physiological action.

EXPERIMENTAL

A rabbit which had been immunised with sSCT for the production of CT-specific antibodies for use in the chicken CT RIA was used in this experiment. Blood was collected into a heparinised tube from the ear vein of the immunised rabbit and also from a non-immunised control rabbit from the same litter: 0.1 ml of undiluted plasma was injected intra-venously into 19 day embryos and blood samples were collected 7.5, 15, 45 or 90 min after the injection. Duplicate 0.1 ml samples of embryo plasma were used for the determination of total calcium concentration. Based on the ability of 0.1 ml of a 1:400 dilution of the antibody containing rabbit plasma to bind approximately 1 ng of sSCT, under the conditions of a RIA, the amount of antibody injected should have been capable of binding up to 400 ng CT. This is more than 8 times the calculated amount of CT circulating in the 19-day embryo , based upon a total ECF volume of about 10 ml and a plasma CT concentration not exceeding 5 ng/ ml.

RESULTS

The results of this experiment are shown in table 5.2.

The injection of 0.1 ml plasma from a non-immunised rabbit had no effect upon the plasma calcium concentration of 19 day chick embryos. When plasma from a sSCT immunised rabbit was used this resulted in a highly significant increase in total plasma calcium concentration after 7.5 min and there was still a significant increase 15 min after the injection. No significant differences between control and experimental groups were detected 45 and 90 min post-injection.

Experiment 12

The correlation of a CT peak with a chronological rather than a physiological stage of development in the chick embryo.

In experiment 2 it was noted that the large peak in CT concentration in chick embryo plasma appeared to correlate well with the pipping stage of development, whether the CT was measured by a RIA or a bioassay. A number of physiological changes take place in the embryo around the time of pipping, mostly associated with the adaptation of the embryo to a

TABLE 5.2

THE EFFECT OF SPECIFIC ANTI-CT ANTIBODIES UPON PLASMA CALCIUM CONCENTRATION IN THE 19-DAY CHICK EMBRYO

TIME MIN	Plasma Calcium Concentration (mM) CONTROLS (non-immunised rabbit plasma)	EXPERIMENTAL (immunised rabbit plasma)
7.5	2.53 ± 0.02 (8)	2.77 ± 0.03^{xxx} (7)
15	2.56 ± 0.04 (8)	2.69 ± 0.03^{x} (7)
45	2.58 ± 0.03 (6)	2.59 ± 0.05^{NS} (6)
90	2.59 ± 0.03 (6)	2.49 ± 0.02^{NS} (6)

x $P < 0.05$

xxx $P < 0.001$

NS non-significant

free-living existence. These changes involve, particularly, the circulatory and respiratory systems, and are associated with marked changes in the composition of the blood. At stage 20A (chorio-allantoic respiration) the pH of the plasma is low (pH 7.39), the P_{CO_2} is high (48.7 mm Hg) and the oxygen tension is low (20.7 mm Hg): at stage 20C (pipping) the pH rises to 7.47, the P_{CO_2} falls to 38.1 mm Hg and the P_{O_2} rises to 40.9 mm Hg (Freeman & Misson, 1970). We wished to investigate whether or not increasing hypoxia or increasing respiratory acidosis might be involved in the stimulation of CT release.

Visschedijk (1968) has shown that the pipping stage can be advanced (5.5 h) or retarded (6.1 h) by covering the shell surrounding the air-sac with wax or by drilling a small hole through the shell to the air-sac, respectively. By using similar methods we wished to investigate whether or not the CT peak was associated with the pipping stage, and thus with the stimuli responsible for pipping.

EXPERIMENTAL

Three groups of 10 fertile eggs were candled at half-hourly intervals to determine the onset of pulmonary respiration (Stage 20B). At this stage the shell surrounding the air sac of an egg was either coated with molten paraffin wax or a hole approximately 2 mm in diameter drilled with a dental drill. A control group of eggs were removed from the incubator at stage 20B and handled but left intact. The time interval between stage 20B and pipping was measured and all pipped embryos were bled by cardiac puncture under light ether anaesthesia. Heparinised plasma was stored under liquid nitrogen prior to the determination of CT by RIA.

RESULTS

The results for this experiment are shown in table 5.3.

The time of pipping was advanced by 5.2 h and retarded by 5.8 h by waxing and drilling the eggs respectively. These results agree closely with those of Visschedijk (1968). The plasma CT concentration of the waxed eggs was lower than the control group but not significantly so, whilst in the drilled eggs the plasma CT had fallen significantly from 4.81 to 3.16 ng/ml sSCT equivalents.

TABLE 5.3

THE EFFECT OF WAXING AND DRILLING ON THE TIME OF PIPPING AND ON THE CONCENTRATION OF CT IN THE PLASMA AT PIPPING.

	Plasma CT (ng/ml sSCT equivalents)	Av. Time Interval (h) (20B - pipping)
Control	4.81 ± 0.39 (10)	9.7
Waxed Eggs	4.05 ± 0.36 (10) NS	4.5
Drilled Eggs	3.16 ± 0.24 (10)^{xx}	15.5

NS Not significant

xx $P < 0.01$

DISCUSSION

Experiment 9 clearly demonstrates that the UB glands of the chick embryo do respond in vivo to a calcium challenge, but it must be noted that relatively large increases in plasma calcium were required to produce a significant increase in plasma CT. Such large increases in plasma calcium were not observed in Experiment 1, although the levels of CT were high, (Experiment 2). This must therefore cast a doubt upon calcium being the natural stimulus for CT release in the chick embryo.

The results of experiment 10 are of considerable interest. The injection of the β-adrenergic blocking agent produced a fall in circulating plasma CT concentration in the 19-day chick embryo and in fact a dose of 1 mg resulted in a fall in CT concentration below detectable limits 1 h after injection. However, in the latter case there was no change in plasma calcium concentration whereas in the dose-response and time course experiments all other doses of propranolol which produce a significant fall in plasma CT were also associated with an increase in plasma calcium concentration. It is still tempting, therefore, to suggest that, in 19 day chick embryos, the action of propranolol is to decrease circulating CT and that, as a consequence, the plasma calcium concentration increases. Furthermore, the fact that 1 mg propranolol 1 h post-injection reduces the circulating CT concentration to undetectable levels indicates that the stimulus for the release of CT in the late embryo is β-adrenergic in nature, either via a neural pathway or via circulating adrenaline.

Lund, Hindberg and Sørensen (1975) suggested that propranolol had a direct action upon plasma calcium concentration to produce a hypocalcaemia, probably by a membrane stabilising effect, but since the effect in chick embryos was the opposite it might therefore be argued that this was indeed the result of an indirect effect via the inhibition of CT secretion. The time taken for the maximum effect to be observed was 60 min and probably represents the time for CT already circulating to be destroyed and removed by enzymes in the plasma, liver and kidney. At 2h post injection the circulating CT is again detectable, which suggests that the effect of propranolol has diminished and CT is again being secreted by the UB glands of the chick embryo.

The involvement of a β-adrenergic stimulatory pathway is also suggested by the action of the β-agonist isoprenaline. Doses of 10 and 1 mg produced a highly significant increase in circulating plasma CT 2 h post injection. However, the control values were undetectable whilst

the control values in the propranolol experiment were in the region of 2 ng/ml plasma. The only difference between these two control groups was that the isoprenaline controls were injected with 0.1 ml 10^{-4} M ascorbic acid at pH 3.0. Clearly this in itself has had a drastic effect but we can find no suitable explanation. There is no evidence that a fall in pH might cause CT secretion other than by increasing the fraction of total plasma calcium found in the ionised form. However, it is doubtful whether the small volume and low molarity of the ascorbic acid solution could have altered the plasma pH sufficiently to completely inhibit CT secretion. The highest dose of isoprenaline certainly produced a 2-fold increase in plasma CT compared to the propranolol controls and we feel justified in suggesting that a direct effect of isoprenaline upon CT secretion from the UB gland occurred.

The effect of isoprenaline upon plasma calcium concentration was dependent upon the dose given. The highest dose, which was probably more pharmacological than physiological, produced a significant hypercalcaemia whilst the two lower doses both produced a significant hypocalcaemia, although the lowest doses did not result in any detectable change in CT. It seems unlikely that these effects of isoprenaline upon plasma calcium are exerted indirectly via a stimulation of CT secretion since

(1) the effect can be hyper- or hypocalcaemic depending upon dose, and appears to be independent of any effect upon plasma CT,

(2) hypocalcaemia occurs without a detectable increase in plasma CT when the lowest dose of isoprenaline is used,

(3) previous experiments have shown that exogenous CT will only have a restricting action upon plasma calcium concentration and that CT has no effect on a normal plasma calcium level in chick embryos.

Plasma inorganic phosphate concentration appeared to be little effected by either propranolol of isoprenaline with the exception that a significant fall from 1.53 to 1.19 mM occurred as a result of the action of 1 mg of isoprenaline. The only point of interest is that the same dose of isoprenaline caused a significant fall in plasma calcium and a highly significant increase in plasma CT. It might have been useful to have observed the effect of this dose of isoprenaline at different time intervals to confirm whether or not this effect was a consistent and physiological action.

The use of specific antibodies to remove circulating levels of polypeptide hormones is a well-established technique. Hedwall (1968) showed that a single intravenous injection of Angiotensin II specific antibodies inhibited the presser effect of Angiotensin II for up to 2.5 h and Malaisse, Malaisse-Lagae and Wright (1967) demonstrated an increased insulin secretion following insulin deficiency induced by a single i.v. injection of anti-insulin antibodies. In experiment 11 the removal of circulating CT clearly resulted in an increase in plasma calcium concentration. This effect was significant for only 15 min but the experiment was limited by the low titre of antibody in the rabbit serum and the small injection volume. The result of this experiment does indicate that the high level of CT found in day 19 embryos was in fact restricting some hypercalcaemic stimulus. This experiment together with the results of experiment 7 which demonstrated the restrictive effect of exogenous sSCT upon an induced hypercalcaemia, gives considerable support to the hypothesis that the role of CT in the chick embryo is to restrict hypercalcaemia.

The probable cause of hypercalacemia in the chick embryo remains unanswered, but the results of experiment 12 suggest that the maximum stimulus to CT secretion in vivo occurs at a specific period of time unrelated to pipping or the stimuli, (mostly respiratory acidosis), which induce pipping. The concentrations of CT in embryos which had pipped 5 h earlier or later than normal correspond closely to values which one would expect 5 h before or after pipping as determined from Fig. 3.3. Certainly the peak of CT has not moved in time so as to coincide with the actual event of pipping.

CHAPTER 6

The experimental work so far presented in this thesis has been related exclusively to the chick embryo and to very young chicks. From this work we have concluded that CT most probably acts in a restrictive manner with respect to plasma calcium homeostasis. The experiments to be reported in this chapter were designed to extend and test this hypothesis in older chicks and in the laying hen. Cipera et al. (1970) demonstrated that an increased calcium content of the diet of young birds, (3-4 weeks) resulted in hypertrophy and hyperplasia of the C-cells of the UB gland although there was only a small change in the CT content of the UB's as estimated by a bioassay. These authors did not measure circulating plasma CT. Bates, Bruce and Care (1969) suggested that the UB glands of geese possessed only minimal CT stores and that a stimulation of the glands increased the CT synthetic rate. Ziegler et al. (1970) confirmed this latter suggestion in the hen. It remained therefore to test the possible role of CT during periods of calcium stress in the chicken by direct measurement of circulating CT.

Experiment 13

The effect of age, sex and dietary calcium upon circulating CT, calcium and inorganic phosphate in young chicks.

An influence of gonadal hormones upon plasma CT and plasma calcium concentrations has been documented by Dacke, Furr, Boelkins and Kenny (1973) in the Japanese Quail and as such the maturity of a chicken would be expected to influence the effect of changes in the amount of calcium in the diet. This experiment was designed to investigate the role of CT in the control of plasma calcium concentration in chickens under a calcium challenge in the form of an increase in dietary calcium. The effects of age, sex and duration of a calcium challenge were incorporated into the experimental design.

EXPERIMENTAL

In a pilot, long term experiment young chicks (ROSS I) were reared on a commercial mash containing 32 g calcium/kg and 7 g phosphorus/kg until four weeks of age. At this time cockerels and pullets were separated and 3-5 birds of each sex were fed either the control diet as above or one supplemented with an additional 30 g calcium/kg (as calcium carbonate). Blood samples (2 ml) were collected by venipuncture from a wing vein of unanaesthetised birds at various time intervals. Heparinised plasma was stored for CT analysis by RIA and plasma calcium concentration was determined by A.A.S. The experiment was terminated when the birds were 26 weeks old and all the pullets had begun to lay.

In a more detailed experiment 100 1 d old chicks (ROSS I) were fed the diet shown in table 6.1 containing 11.4 g calcium/kg and 9.4 g phosphorus/kg for five weeks. At this time both cockerels and pullets were separated into three groups of 14-16 and fed either the diet shown in table 6.1 or this diet supplemented with an additional 20 or 50 g calcium/kg(as calcium carbonate) to give three groups with a total dietary calcium content of approximately 10, 30 or 60 g/kg. At the commencement of the experimental period the birds were transferred to deep litter cages and food and water were available ad libitum.

After 1, 3 and 5 weeks of the experiment all birds were bled (2 ml) as above and the plasma CT, calcium and inorganic phosphate were determined by RIA, AAS and autoanalysis respectively.

RESULTS

Results for these experiments are shown in table 6.2 and Figs. 6-1, 6-2 and 6-3.

LONG TERM EXPERIMENT (62g/kg calcium diet commenced at wk 4) (Table 6.2)

(a) Cockerels

(1) 32 g calcium/kg resulted in a high plasma calcium concentrations (3.6 mM) from wks 7-18 which thereafter fell to a value of 2.9 mM by wk 26. CT was detectable at all times but there was no correlation between plasma CT and calcium concentrations.

TABLE 6.1

BASIC CHICK DIET

Maize Meal	500 g/kg	
Soya Bean Meal	353 " "	
Dried Yeast	50 " "	(Providing 0.8 g/kg available phosphorus)
Corn Oil	50 " "	
Methionine	1 " "	
$CaHPO_4.2H_2O$	43 " "	(Providing 20 g/kg calcium 8 g/kg phosphorus)
NaCl	3 " "	
Rovimix A-D†	4-6 mg/kg	
ZnO	120 " "	
$MnCO_3$	150 " "	

† contains 2000 i.u.A and 400 i.u.D /5 mg

Calcium and Phosphorus estimations by A.A.A and Autoanalysis of acid digested diet.

11.4 g calcium/kg

9.4 g phosphorous/kg

(2) 62 g calcium/kg resulted in a very high plasma calcium concentration (4.13 mM) in 7 wk old cockerels but thereafter the plasma calcium concentration fell to 2.83 mM by wk 26. The highest CT level found at wk 7 was significantly greater than all other values in the 62 g/kg group and from the CT level found at wk 7 in cockerels fed the 32 g/kg diet. From weeks 18-26 however, the plasma CT concentration tended to be less in the 62 g/kg compared to the 32 g/kg group at each time of sampling, but not singificantly so.

(b) Pullets

(1) 32 g calcium/kg resulted in a plasma calcium concentration of 2.75 mM at wk 7 which was significantly lower than the equivalent cockerel group at the same age of sampling. Thereafter the plasma calcium concentrations were very high, but this was due to the onset of laying. The plasma CT concentrations varied between 2.15 ng/ml of wk 7 to 1.28 ng/ml at wk 26 when all the pullets in the group had begun to lay. Similar to the equivalent cockerel group the CT values did not correlate well with the calcium concentrations found.

(2) 62 g calcium/kg resulted in a plasma calcium concentration of 3.68 mM at wk 7 which was significantly greater than the 32 g/kg pullet group at the same age of sampling but was less than the equivalent cockerel group but not significantly so. This high plasma calcium concentration was associated with a high CT value (2.75 ng/ml) but again this value, whilst being greater than the 32 g/kg pullet group at wk 7 was significantly less than the equivalent cockerel group. As in the 32 g/kg pullet group the plasma calcium values in the 62 g/kg group from wks 18-26 varied enormously (4.1 - 7.5 mM) without apparently producing any large changes in circulating plasma CT.

SHORTER TERM EXPERIMENT

(1) The effect of dietary calcium upon plasma calcium (Fig. 6.1).

TABLE 6.2

CHANGES WITH AGE IN THE EFFECT OF DIETARY CALCIUM UPON PLASMA CALCIUM AND CALCITONIN IN COCKERELS AND PULLETS

COCKERELS

Dietary Ca	32 g/kg		62 g/kg	
Age (Wk)	CT	Ca	CT	Ca
7	2.44±0.16	3.65±0.05 (3)	4.13±0.36^{xx}	4.13±0.13 (4)^{x}
18	1.83±0.22	3.58±0.30 (3)	1.50±0.24	3.85±0.18 (3)
20	2.63±0.36	2.73±0.01 (3)	1.81±0.34	3.33±0.15 (3)
22	2.11±0.33	2.95±0.08 (3)	1.36±0.11	3.43±0.30 (3)
26	1.84±0.29	2.90±0.05 (3)	1.65±0.06	2.83±0.18 (3)

PULLETS

Dietary Ca	32 g /kg		62 g/kg	
Age (Wk)	CT	Ca	CT	Ca
7	2.15±0.17	2.75±0.13** (5)	2.75±0.19**	3.68±0.25 (4)^{xx}
18	1.34±0.16	5.68±0.83 (5)	1.54±0.19	4.10±0.45 (4)
20	1.48±0.08	5.15±0.63 (5)	1.62±0.14	4.40± 1.48 (4)†
22	1.48±0.06	6.55±0.05 (4)‡	2.14±0.61	7.45±0.25 (4)‡
26	1.28±0.25	5.58± 1.03 (3)‡‡	1.54±0.02	5.85±0.70 (3)‡‡

† 1 pullet egg laying

‡ 2 pullets egg laying

‡‡ All pullets egg laying

x $P < 0.05$ cf. 3.2% group

xx $P < 0.01$ cf. 3.2% group of same sex

** $P < 0.01$ cf. equivalent cockerel group

CT Calcitonin concentration in plasma (ng/ml sSCT equivalent)

Ca Plasma calcium concentration (mM)

Percentage figures in parentheses refer to the calcium content of the diet. The high calcium diet (6.2%) commenced at 4 weeks of age.

(a) Cockerels

(1) Increasing the dietary calcium from 10 to 30 g/kg resulted in an increase in plasma calcium concentration but which was only significantly different after 1 week of the experiment (2.54 to 2.61 mM).

(2) Increasing the dietary calcium from 10 to 60 g/kg resulted in highly significant increases in plasma calcium at all times of sampling (2.55 mM to 2.74-2.81 mM).

(b) Pullets

(1) A 30 g calcium/kg diet significantly increased plasma calcium concentration at 3 and 5 weeks but unlike the cockerel group there was no significant increase over the 10 g/kg group at 1 week.

(2) The effects of a 60 g calcium/kg diet were virtually identical to those in the cockerel groups i.e. highly significant hypercalcaemia at all times of sampling.

(2) The effect of dietary calcium upon plasma phosphate (Fig. 6.2)

(a) Cockerels

(1) Plasma inorganic phosphate varied inversely to the dietary calcium content at all three times of sampling falling from a value of about 2 mM to the low value of 1.1 mM when the dietary calcium increased from 10 to 60 g/kg.

(b) Pullets

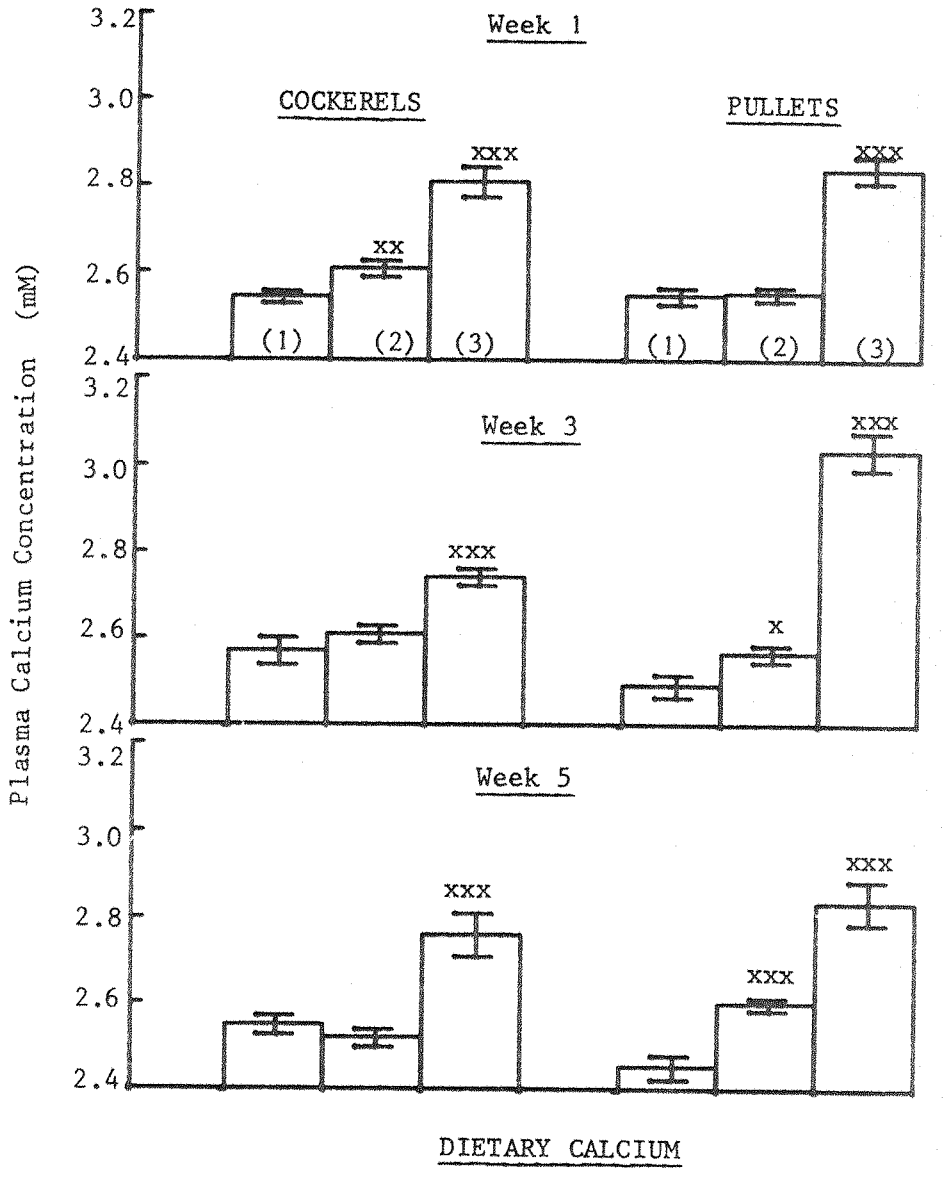
(1) Plasma inorganic phosphate values were similar to the cockerel groups, although the effect of a 30g calcium/kg diet at weeks 3 and 5 was very small and not significant. The fall in plasma phosphate induced by the 60 g calcium/kg diet was highly significant at all three times of sampling.

(3) The effect of dietary calcium upon plasma CT (Fig. 6.3).

Cockerels and Pullets

(1) Generally there were small increases in plasma CT as the dietary calcium content was increased,but by week 5 this effect was very small and only significant for the 60g/kg pullet group.

FIG. 6.1 The effect of dietary calcium upon plasma calcium in young cockerels and pullets



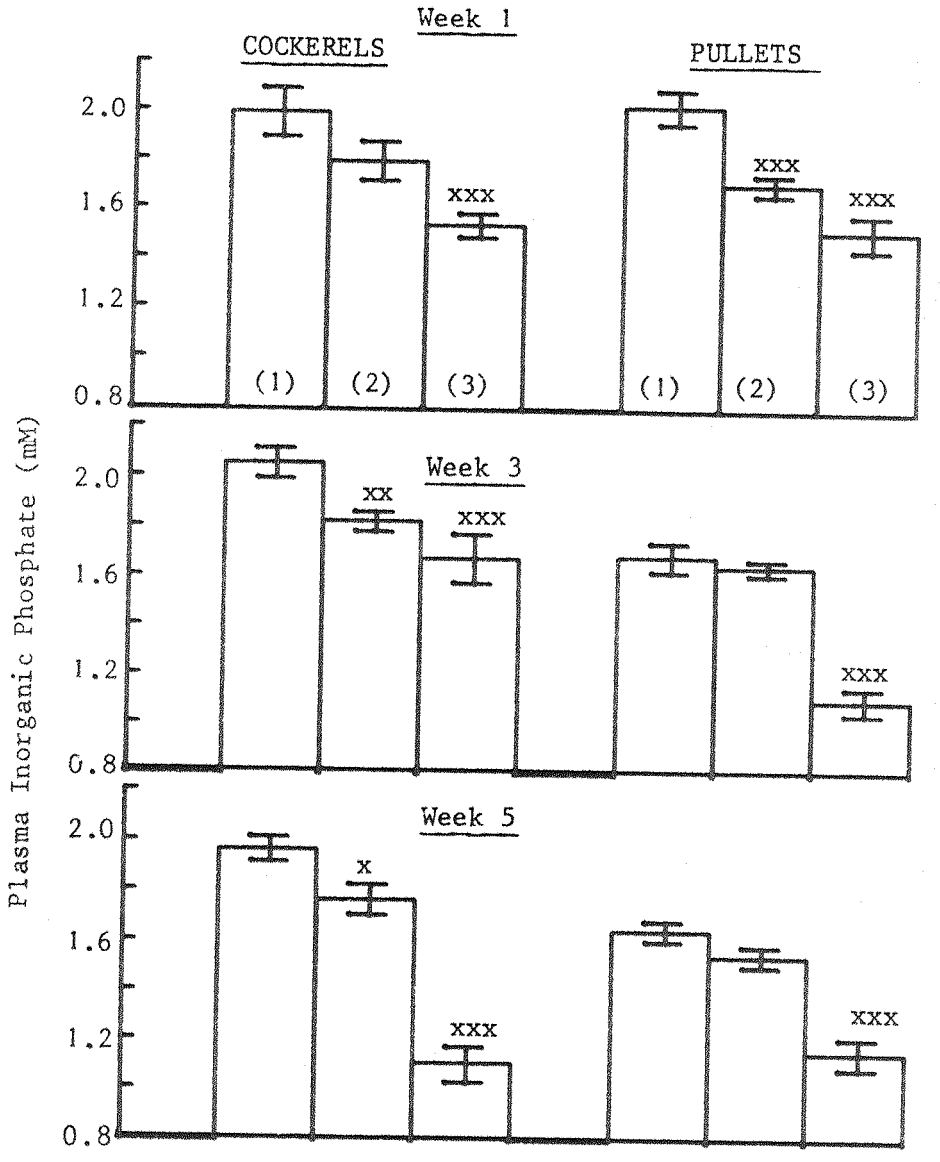
(1) 10 g/kg (2) 30 g/kg (3) 60 g/kg

n = 16 for each group except for 30 g/kg cockerel group where n = 14

x $P < 0.05$ xx $P < 0.01$ xxx $P < 0.001$

compared to the control (10 g/kg) group in each case

FIG. 6.2 The effect of dietary calcium upon plasma inorganic phosphate in young cockerels and pullets



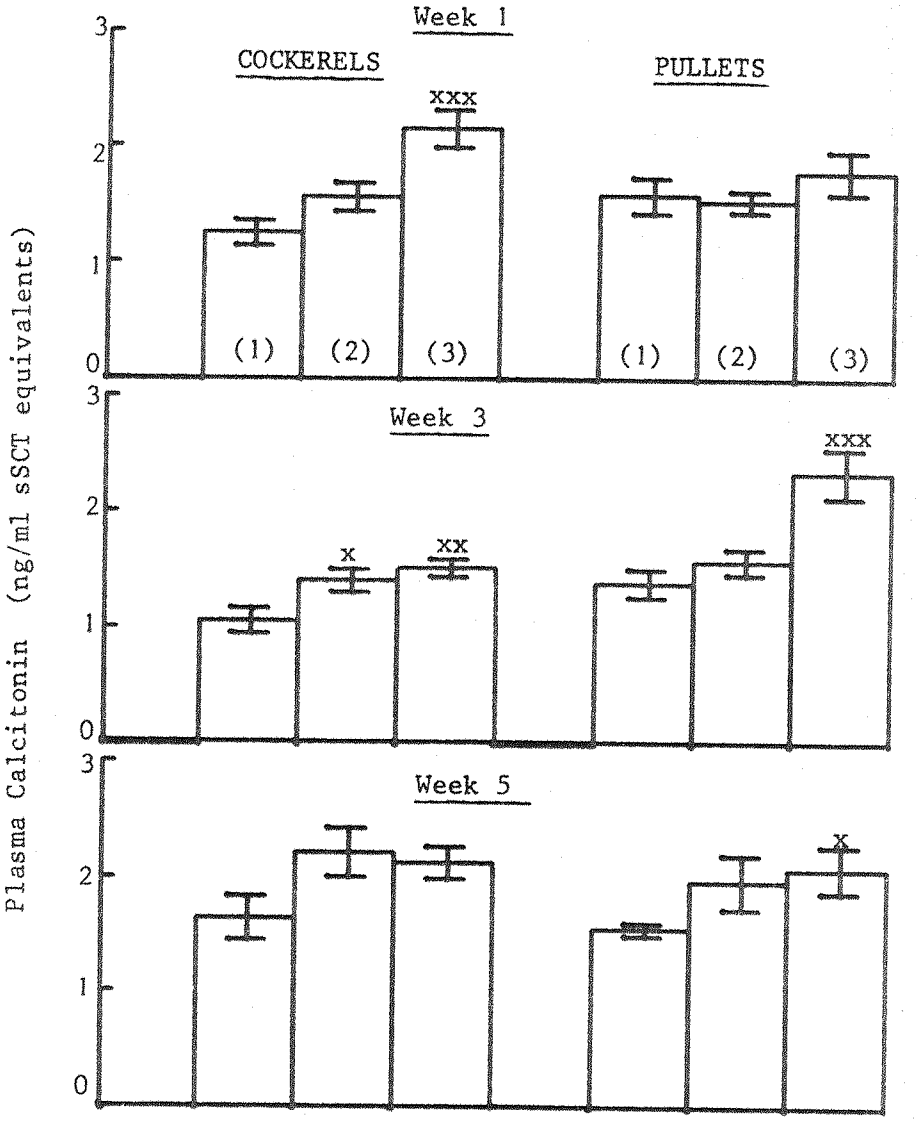
(1) 10 g/kg (2) 30 g/kg (3) 60 g/kg

n = 16 for each group except for the 30g/kg cockerel group where n = 14

x $P < 0.05$ xx $P < 0.01$ xxx $P < 0.001$

compared to the control (10 g/kg) group in each case

FIG. 6.3 The effect of dietary calcium upon plasma calcitonin concentration in young cockerels and pullets



(1) 10 g/kg (2) 30 g/kg (3) 60 g/kg

n = 16 for each group except for the 30 g/kg cockerel group where n = 14

x $P < 0.05$ xx $P < 0.01$ xxx $P < 0.001$

compared to the control (10 g/kg) group in each case

(2) The control (10 g calcium/kg) groups all had a detectable circulating CT level in the range of 1.05 - 1.64 ng/ml sSCT equivalents.

DISCUSSION

Garlich and Bryant (1975) studied the effect of dietary calcium and phosphorus upon plasma calcium concentration in intact and UBX cockerels at 17 weeks of age. They concluded that "the presence of the ultimobranchial glands are essential to the regulation of plasma calcium and phosphorus in chickens which consume high calcium - low phosphorus diets". They did not however attempt to measure circulating CT, nor did they use very young cockerels or pullets. In addition, only total plasma calcium values were quoted, not ionic concentrations, although this is also a criticism of all the experiments recorded in this thesis.

The long term experiment suggested that both cockerels and pullets respond to an increase in dietary calcium by increasing circulating CT. This effect was only noted in 7 week old cockerels and pullets which had been exposed to a high calcium diet for 3 weeks. At all later times of sampling, the cockerels were still hypercalcaemic but the CT levels for both 32 g/kg and 62 g/kg groups were very similar. It should be noted that the lowest (32 g/kg) calcium diet used would be considered by most authors to be excessively high in calcium for growing birds and corresponds to the diet required by a laying hen.

In the case of the pullets in the long term experiment, the plasma calcium concentrations were already high at 18 weeks, in preparation for laying. The effect of increasing dietary calcium from 32 to 62 g/kg is difficult to determine due to the large variation in individual plasma calcium concentrations within the same dietary group. However, there is clearly a fall in CT levels which is maintained from weeks 18 - 26 compared to week 7. In laying hens the blood sample was collected randomly and not at any particular time of the egg-laying cycle.

After three weeks of the experimental diet it is interesting that the mean concentration of CT in the cocks was significantly greater than in the pullets. This is in agreement with Dacke, Boelkins, Smith and Kenny (1972) and Dacke, Furr, Boelkins and Kenny (1973) who found that CT levels in mature male Japanese Quail were up to three times higher than levels found in the female quail. These authors also found

that CT in chickens were much lower than in quail and were often undetectable by the bioassay method they employed. The values for CT concentration reported in experiment 13 ranged from 1-4 ng/ml which is equivalent to 4,790 - 19,160 μu/ml, whilst the highest levels reported by Dacke et al were in the order of 1000 μu/ml in male quail. Our values fall more into the levels observed in spawning Chinook salmon by Copp et al (1972), which were 10,000 and 67,000 μu/ml for male and female respectively.

The transient nature of the CT response to an increase in dietary calcium in both young cockerels and pullets may well be explained by an adaptation in the rate of calcium transport by the jejunum, which according to Hurwitz and Bar (1966) is the major calcium absorbing site in the chicken. However, whilst the calcium transport may be reduced it would appear that such a mechanism is insufficient to totally prevent plasma hypercalcaemia, which is present from weeks 18-26 in the 62 g/kg cockerel group, although the CT levels were much lower (< 2 ng/ml) throughout this period. Food intake was not measured in this experiment.

As noted previously the lowest calcium diet used in the long term experiment was 32 g/kg which appeared to be sufficient to maintain detectable levels of CT in all the experimental groups. The short term experiment was designed to include a "normal" calcium group (11.4 g/kg) and changes in plasma calcium, phosphate and CT resulting from increases in dietary calcium to 30 and 60 g/kg were examined after 1, 3 and 5 weeks on experimental diets which commenced when the birds were five weeks of age.

The plasma calcium concentration of cockerels appeared to adapt rapidly to an increase in dietary calcium from 10 to 30 g/kg but the increase to 60 g calcium/kg resulted in a highly significant hypercalcaemia at all three times of sampling. Particularly associated with the high calcium levels resulting from a diet containing 60 g calcium/kg was a significant fall in the concentration of inorganic phosphate in the plasma. CT levels in the cockerel groups were also increased after 1 and 3 weeks on the 60 g/kg diet but by week 5 the CT levels were not significantly different as a result of the different dietary calcium. Certainly the changes in plasma CT were not striking and the presence of detectable levels even in the 10 g/kg group was surprising.

The effect of dietary calcium in pullets was very similar to the effect in cockerels i.e. an increase in plasma calcium associated with a decrease in plasma inorganic phosphate, particularly in the 60 g/kg group, but little effect upon plasma CT levels. Again CT was detectable in the 10 g/kg group.

The food intake was not measured, but ample supplies of food and water were available at all times although presentation of fresh food(which occurred between 9 and 10 am each day) always resulted in a rush of feeding activity. Since samples were taken from both cockerels and pullets in the post feeding period (morning) the results may have been affected by the digestion of the recently ingested food. A role for CT in post-prandial control of hypercalcaemia is now well established in the pig (Care et al, 1974) but little work has been done to confirm whether or not such a role for CT exists in the chicken. One could however speculate that the detectable levels of CT even in the 10 g calcium/kg group may have been due to the eating habits of the birds and not necessarily to the level of dietary calcium. One argument against this possibility is the fact that birds are not meal eaters, and the presence of the crop ensures that food passes through the gastro-intestinal tract continuously (except in the early hours of morning before feeding begins, when the crop is empty).

At the end of the five weeks of the experiment the body weights of the 6 experimental groups were recorded and are shown below;

	Dietary Calcium (g/kg)		
Body Wt. (g)	10	30	60
Cockerels	1558 ± 35 (16)	1497 ± 16 ^{NS}(14)	1394 ± 16 ^{xx}(16)
Pullets	1049 ± 25 (16)	1078 ± 38 (16)	1023 ± 18 (16)

xx $P < 0.01$

NS Not significant

It is interesting that the cockerels, but not the pullets, appear to have reduced their dietary intake significantly when fed a 60 g calcium/kg diet.(No other explanation for the 10% fall in body weight during the course of the experiment seems reasonable). In the experiments performed by Garlich and Bryant (1975) no such adaptation in food intake or body weight was observed. However, the latter authors

commenced experimental periods when the cockerels were 16 weeks old whereas our experimental period was completed at 10 weeks of age.

In conclusion it should be emphasised that increasing dietary calcium resulted in

(1) an increase in plasma calcium concentration

(2) a decrease in plasma inorganic phosphate

(3) small increases in plasma CT concentration

in both cockerels and pullets particularly in the short term, although there does appear to be an adaptation to the diet either by decreasing the efficiency of calcium absorption in the gut and/or decreasing food intake (cockerels only). The role of CT does not appear to be striking, although the presence of high levels of CT even in the 10 g calcium/kg groups suggests that other factors may be involved e.g. post-prandial secretion of CT following feeding. Finally there is some evidence that young cockerels may be more sensitive to increases in dietary calcium but this was only noticeable in the long term experiment.

Experiment 14

Changes in calcium CT, and inorganic phosphate in the plasma of domestic fowl during the ovulatory cycle.

A naturally occurring period of calcium stress prevails in the chicken during the egg-laying cycle and we wished to investigate changes in circulating plasma CT at different times of this cycle. Previous experiments (Dacke, Furr, Boelkins and Kenny, 1973) with Japanese quail suggested that CT may be involved in the prevention of hypercalcaemia at periods in the ovulation cycle when little egg-shell calcification is taking place. Certainly there are fluctuations in both total and diffusible calcium associated with shell-calcification (Hertelendy and Taylor, 1961; Taylor and Hertelendy, 1961) and the demand for calcium during the calcification is enormous, representing approximately 10% of the total calcium content of the hen.

The physiological adaptations relating to the calcium metabolism of the hen in preparation for the onset of laying are firstly the formation of a unique labile form of bone, medullary bone, (Taylor, 1970), secondly an increase in the efficiency of calcium absorption in the intestinal tract (Hurwitz and Bar, 1969) and thirdly an enormous increase in total plasma calcium, largely resulting from high circulating levels of calcium binding plasma proteins which facilitate calcium transport to the ovary.

EXPERIMENTAL

A medium hybrid strain (ROSS) of hen was used in this experiment. Blood samples (2 ml) were collected from unanaesthetised hens within 15 mins of oviposition and further samples were collected every 4 h until the sixth sample which was taken at 20 h. After this time the hens were observed every 30 mins and the time of the second oviposition was recorded. Any hen which did not lay a second egg within 36 h was checked at regular (approx. 6 h) intervals until a second egg was produced.

Heparinised plasma was used for CT, calcium and inorganic phosphate determination by RIA, AAS and autoanalysis respectively. Before, during and after the experimental period a commercial mash, (containing 32 g calcium/kg and 7 g phosphorus/kg), and water was available ad libitum. The hens were housed individually in commercial battery cages with a 14 h artificial light 10 h dark cycle.

RESULTS

The results for each individual hen are shown in table 6.3.

For the purpose of the following description of results and subsequent discussion the hens were grouped according to the time interval between the first and second ovipositions.

Hen No's 2 and 3 (24.8 and 24.5 h respectively)

In these two hens CT was detectable at the time of the first oviposition, fell to a lower level at 4 h and thereafter was undetectable (< 0.5 ng/ml). Plasma calcium concentration varied a little but there was no obvious fall in total calcium concentration at the time in the ovulatory cycle corresponding to the period of rapid egg-shell calcification (10 - 25 h). Plasma inorganic phosphate increased in the middle of the cycle but was low just after and 5 h before oviposition.

Hen No's 1 and 8 (34.8 and 38 h respectively)

In these two hens CT was detectable (and high) at the time of the first oviposition and remained detectable at 4, 8 and 12 h following oviposition. At 16 and 20 h CT was no longer detectable (< 0.5 ng/ml), the plasma calcium concentration remained fairly constant with the exception of the final time of sampling (20 h) when there was a fall in total plasma calcium concentration in both hens. The plasma inorganic phosphate increased with time in hen 1 but fluctuated considerably in hen 8.

TABLE 6.3

CALCIUM, CALCITONIN AND INORGANIC PHOSPHATE DURING THE EGG-LAYING CYCLE OF THE HEN.

TIME OF FIRST OVIPOSITION	HEN No.		TIME AFTER FIRST OVIPOSITION (h) 0	4	8	12	16	20	INTERVAL BETWEEN FIRST AND SECOND OVIPOSITION (h)
0930	1	CT	1.47	1.8	0.75	1.0	–	–	
		Ca	4.58	4.70	4.70	4.65	4.65	4.10	34.8
		PO_4	1.15	1.21	1.14	2.04	2.32	2.42	
0945	2		1.22	0.74	–	–	–	–	
			5.25	5.58	5.32	5.13	5.45	5.50	24.8
			0.73	0.93	1.12	1.14	1.68	1.13	
1135	3		1.9	0.66	–	–	–	–	
			5.53	6.25	5.95	5.80	5.68	5.63	24.5
			0.72	1.30	1.32	1.39	1.12	0.51	
1140+	4		1.33	–	–	–	–	–	
			5.53	5.45	5.88	5.23	4.65	5.75	48.54 (Soft Shell)
			0.77	0.73	1.72	1.28	1.20	1.61	
1200	5		–	–	–	–	–	–	
			6.05	8.23	6.30	6.13	5.23	5.13	> 48
			1.37	1.78	2.12	1.80	1.44	1.38	
1205	6		0.95	–	–	–	–	–	
			5.00	5.50	5.73	6.53	5.35	4.75	> 48
			1.28	1.75	1.96	1.65	2.12	0.97	
1230	7		–	–	–	–	–	–	
			5.63	5.88	5.63	5.90	4.78	5.50	> 48
			1.15	1.18	1.12	0.93	0.88	0.94	
1240	8		2.48	2.31	2.45	1.12	–	–	
			4.80	5.05	5.00	5.35	4.78	4.30	38
			1.11	0.95	0.78	2.84	1.43	1.11	
1425	9		–	–	–	–	–	–	
			5.78	6.35	5.20	5.48	4.63	4.63	> 48
			1.31	2.32	1.47	1.51	1.33	0.98	
1510	10		1.21	–	–	–	–	–	
			5.75	5.95	5.50	4.65	4.20	5.00	> 48
			1.40	2.33	1.48	1.27	1.19	0.74	
1515	11		–	–	–	–	–	–	
			7.83	7.93	8.25	6.70	6.88	5.50	> 48
			1.66	2.60	1.95	1.44	1.32	1.58	

CT as ng/ml sSCT equivalents. A space denotes an undetectable level (< 0.5 ng/ml)
Calcium - mM
Inorganic Phosphate - mM
+ Soft-shelled egg.

Hens 4, 5, 6, 7, 9, 10 and 11

All these hens failed to lay a second egg within 48 h and in no case was CT detectable after the 0 h stage. Both eggs laid by hen 4 were soft-shelled. Generally plasma calcium and phosphate fluctuated a little, although the plasma cal ium values found in hen 10 produced a pattern which normally would be associated with egg-shell calcification and that in hen 11 fell progressively after 8 h from the high value of 8.25 mM to 5.50 mM at 20 h.

DISCUSSION

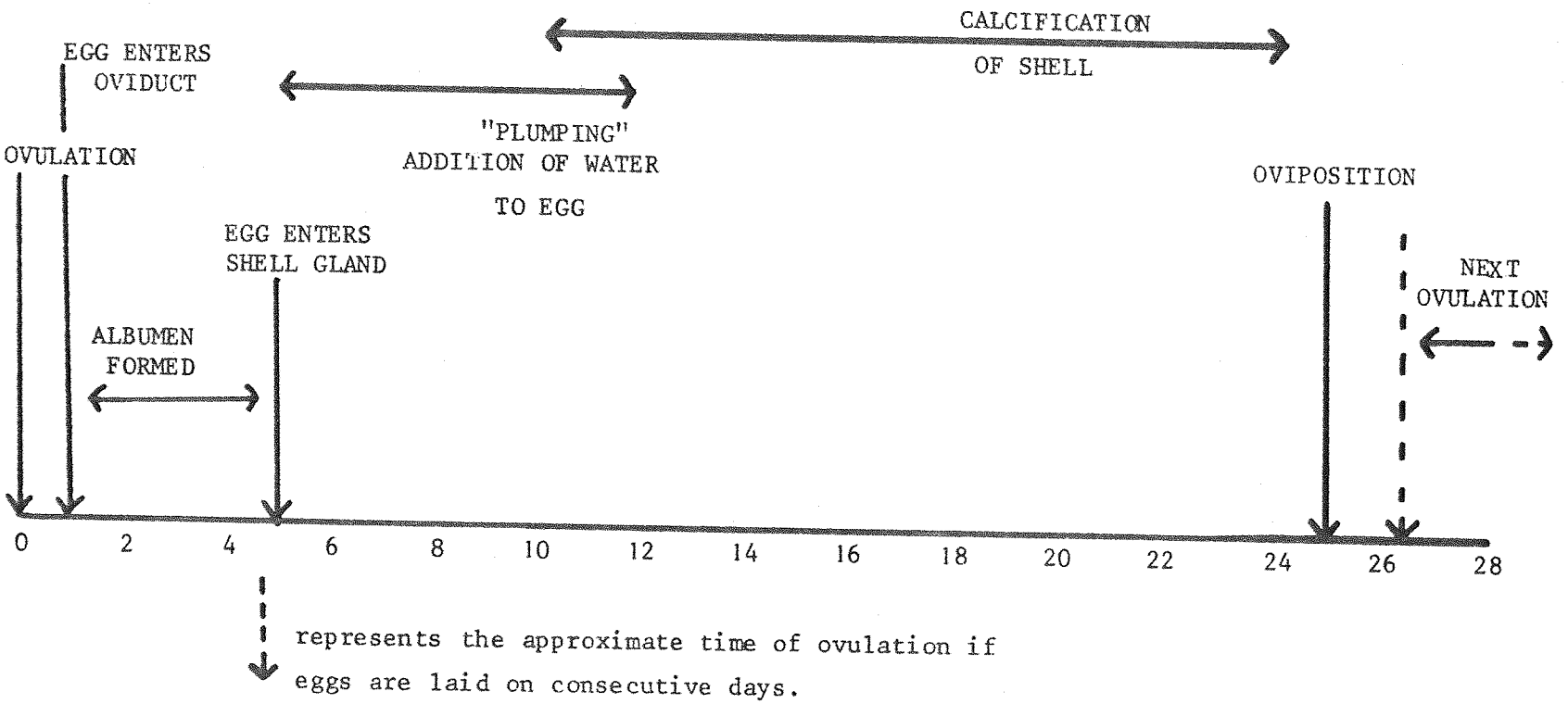
Generally the results of this experiment support the view that CT may play a role in plasma calcium homeostasis during the ovulatory cycle of the hen. In particular high levels of CT are detectable in the plasma of hens between oviposition and the start of egg-shell calcification. A diagrammatic representation of the ovulatory cycle is presented in Fig. 6.4.

In the two hens which laid a second egg within 26 h, CT was detectable at 0 and 4 h. In the two hens which laid a second egg at about 34-38 h CT was detectable for 12 h, presumably because the period between the first oviposition and the calcification of the second egg had been extended. In the remaining birds a second egg was not laid within 48 h, and with three exceptions CT was undetectable.

During the calcification of an egg-shell, the rate of utilisation of plasma calcium is very high and probably relieves somewhat the calcium stress placed upon plasma calcium homeostasis due to the rapid resorption of medullary bone and the increased efficiency of calcium transport in the intestine. It is most likely that the cessation of calcification, providing it is followed by a second ovulation, exerts a significant hypercalcaemic stimulus, in view of the fact that calcium resorption and transport rates would still be high but the available calcium would not be utilised in shell calcification.

When a second ovulation is delayed the period of hypercalcaemia may well be extended and indeed CT levels were found to be high for a longer period of time in these birds (1 and 8). The CT results for birds that failed to ovulate a second time suggest that there was a rapid decline in medullary bone resorption and a fall in the efficiency of calcium transport in the gut resulting in a much reduced calcium input

FIG. 6.4 Diagrammatic representation of the development of the egg subsequent to ovulation †



† after Gilbert 1971

into the plasma coincident with a period of non-egg-shell calcification, thus eliminating a hypercalcaemic stimulus and the requirement for CT.

The most common reason for the laying of a soft-shelled egg is premature expulsion of the egg from the shell-gland. If this was the cause in hen 4 then the presence of CT at the time of the first oviposition may be the result of a hypercalcaemic stimulus resulting from the non-utilisation of calcium for the formation of the egg-shell. It would seem though that the normal mechanisms which maintain calcification of the egg-shell for up to 16 h are inhibited fairly rapidly, since 4 h after oviposition in hen 4 CT is no longer detectable, and it is tempting to suggest that CT itself was such an inhibitory factor. The transient appearance of CT in two other hens which did not lay a second egg within 48 h of the first may similarly be the detection of such a "switching off" mechanism of the calcium mobilising factors.

Bloom, Domm, Nalbandov and Bloom (1958) studied medullary bone in the laying chicken and associated well-defined histological patterns with the onset of shell formation and the time at which shell formation was well advanced. The formation of medullary bone under the influence of oestrogens acting in synergism with androgens is well documented and has been reviewed by Simkiss (1961, 1967) and Taylor and Stringer (1965). However, the mechanism by which medullary bone is resorbed has been the subject of much discussion. Riddle, Rauch and Smith (1945) advanced an "oestrogen withdrawal" theory which depended upon two propositions. The first was that levels of oestrogens in the blood of laying birds fell rapidly after ovulation, resulting in the resorption of medullary bone calcium which is then utilised in egg-shell calcification. The second requires that such a fall does in fact lead to resorption of medullary bone. This suggestion was supported by Urist (1959) and Urist, Deutsch, Pomerantz and McLean (1960). Only recently has the direct measurement of circulating oestradiol in the plasma of laying hens at different times of the ovulatory cycle been achieved, (Senior and Cunningham, 1974). Their results show high levels (150 pg/ml) of oestradiol 6 h before oviposition and the lowest levels (75-80 pg/ml) were found 12 and 10 h and at oviposition. Clearly the highest levels were found at a period of rapid egg-shell calcification and does not support the "oestrogen withdrawal" theory.

The alternative theory of medullary bone resorption has been advanced by Taylor (1965 and 1970), Taylor and Belanger (1969) and suggests that parathyroid (PTH) hormone is the inducing factor. This theory unifies the resorption of both ordinary bone and medullary bone but PTH has not as yet been determined in the plasma of laying chicks. The well known role of CT in the inhibition of PTH bone resorption in mammals and the detection of CT at times in the ovulatory cycle when inhibition of medullary bone formation would be advantageous does suggest that these two hormones may well control the rate of resorption of medullary bone.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

The experiments reported in this thesis have already been discussed in some detail but the aim of this general discussion is to corrleate the results and conclusions from the different experiments and from those of other authors, and to draw some general conclusions regarding the role of calcitonin in the control of calcium homeostasis in the chick embryo and chicken. In addition it will be necessary to speculate to a certain extent whenever certain critical points have not as yet been supported by experimental evidence or where the reported literature is confused, in order to present as complete a view as possible. It is recognised that in this respect we may at times be in error but our judgement of some controversial points can only be based upon experimental evidence to date.

The initial stimulus for the work presented in this thesis arose from the observation by Taylor and Lewis (1972) that high levels of CT are found quite naturally in the plasma of chick embryos in the late stages of incubation. At this time the role of CT in non-mammalian species was (and still remains) little understood. An investigation of the factors stimulating the release of CT and its subsequent effects was therefore begun in an attempt to elucidate the function of CT in the chick embryo.

The association of CT with calcium metabolism since its discovery in 1961 naturally led us to examine the changes occurring in the concentration of calcium (and other associated ions) in the plasma of chick embryos, and, as reported in experiment 1, it was found that whenever CT was present in the plasma there persisted a mild hypercalcaemia. It must be stated that a major criticism of this experiment (and all others in which plasma calcium values are reported) is that only the total plasma calcium value was measured, not the physiologically important ionic calcium. Whilst it is true that in adult, stable animals the relationship between ionic and total calcium is an equilibrium system and generally a change in total calcium is indicative of a change (in the same direction) in the ionic fraction, this may not always be the case. One factor which may affect the fraction of the ionic calcium is the total protein content of the plasma. In the embryo

this value doubles between day 14 of incubation and hatching whilst the total calcium varies but a little, (Protein, 12.5 - 24.7 g/l, Romanoff 1967; calcium 2.42 - 2.62 mM) and it is therefore possible that the ionic calcium concentration is high in young embryos but then decreases progressively. Little correlation between changes in magnesium or inorganic phosphate with the CT peak could be observed.

Two questions arise from the measurement of plasma calcium concentration:

(1) Is the degree of hypercalcaemia observed sufficient to cause the stimulation of CT release

(2) Has the circulating CT had any effect upon plasma calcium.

With regard to the first point it might be argued that the possible high ionic calcium concentration first stimulates CT release at day 17 but the plasma protein concentration is increasing continuously and by hatching is sufficiently high to reduce the fraction of ionic calcium and thus stop the stimulation of CT. However, between stages 20A and 20C the plasma CT concentration almost doubles whilst little change in plasma protein concentration can have taken place. Other factors, notably pH, may be of importance in determining the fraction of ionic calcium but between stages 20A and 20C the pH increases (Freeman and Misson, 1970) and presumably results in a fall in ionic calcium, not an increase. In terms of total calcium it is unlikely that the degree of hypercalcaemia observed could be responsible for the release of such large amounts of CT and again the total calcium plateaus between stages 20A and 20C whilst the CT continues to increase. In this respect the results of experiment 9 are relevant since it was shown that the UB glands do respond to a calcium challenge by releasing CT but the degree of hypercalcaemia required is quite severe and much greater than any change in total calcium observed naturally. It would therefore seem to be unlikely that changes in plasma calcium concentration can account for the changes in CT although high ionic calcium concentrations around day 17 may, at least in part, be responsible for the initial stimulation.

With regard to the second question it would seem that the high CT correlates with a fall in plasma calcium only after stage 20C. Prior to this time there are periods when CT is continuing to increase whilst plasma calcium is also increasing or has reached a plateau level. It seems to us that the most likely function of CT is to restrict a hypercalcaemic stimulus. Whatever this stimulus may be it would appear

to increase progressively up to the time of pipping and then decrease rapidly. That CT can act in a restrictive role in birds was first shown by Copp et al 1970 and the results of experiment 11 convincingly suggest that CT may function as a calcium restricting factor in the chick embryo. Clearly shown in this experiment was the hypercalcaemic effect of CT-specific antibodies. Further support for a restrictive role for CT emerged from the results of experiment 7 in which CT was demonstrated to restrict artificially induced hypercalcaemia. In our view the simple design of these two experiments together with the use of physiological amounts of CT enhances the confidence with which we would assert our belief that not only can CT act in a restrictive role but that this is a function of the naturally occurring CT in vivo in intact chick embryos. Further, we would assert that a factor(s) is present from day 17 of incubation and until just after hatching which would, were it not for the restrictive role of CT, result in hypercalcaemia.

Clearly, from the above statements, it is necessary to speculate as to the nature of the factor(s) responsible for stimulating CT secretion and the following possibilities are put forward

(1) PTH/calcium
(2) Gastro-intestinal hormones
(3) Adrenaline (systemic or via a neural pathway)
(4) Hypoxia/Respiratory acidosis.

Any hypothesis that seeks to explain the high concentration of plasma CT in terms of a response to an equally high concentration of PTH must also offer an explanation for the release of PTH. Hunt and Perris (1973) have reported that erythropoietin causes hypercalcaemia in rats, provided that the parathyroid glands are intact, and it is also possible that secretion of this hormone was responsible for the hypercalcaemia observed in the embryos. Freeman and Misson (1970) have shown that the oxygen tension of the blood of chick embryos during the 48 h period before the air-cell is ruptured is very low (20-22 mm Hg), and this could provide a stimulus to erythropoietin (and subsequently PTH) secretion.

Another possible cause of PTH stimulation may be the loss of blood supply to the chorio-allantoic circulation which occurs towards the end of incubation. Experiments 3, 4 and 5 all support the hypothesis that the yolk-sac acts as a calcium reservoir in the middle stages of incubation and this source of calcium is only utilised in the late stages.

Presumably the process by which calcium is removed from the yolk-sac is controlled and it would not be unreasonable to suggest that PTH is required for the transport of calcium from the yolk-sac to the embryonic circulation. If this is the case then the stimulus would be expected to be hypocalcaemia resulting from the continued removal of calcium from the embryonic circulation to the calcifying skeleton at a time when the chorio-allantoic source of calcium becomes limiting. However there is little temporal agreement between the onset of CT stimulation and the reduction of calcium transfer from the shell, nor is there any evidence of hypocalcaemia around the day 19-20 stage of incubation. The "switch-over" from shell to yolk-sac undoubtedly occurs but the utilisation of yolk calcium may simply follow passively the utilisation of other yolk-sac nutrients by the rapidly growing embryo.

A more likely stimulus for CT release is the stimulation of gastro-intestinal (GI) hormones. Experiment 4 has shown that whilst albumen mixed with allantoic fluid is actively ingested from day 14 of incubation (Romanoff, 1960) when it is labelled at day 12 with ^{45}Ca little of this isotope enters the embryo or yolk until day 18. By the time the chick hatches virtually all of the injected dose of ^{45}Ca is accounted for in the ash of the newly hatched chick and its "spare" yolk. It would seem therefore that the intestinal tract becomes functional at about the time that CT first appears. A gastro-intestinal thyroid C-cell system has been well established in the pig by Swaminathan et al (1973) and it has been assigned a role in the prevention of post-prandial hypercalcaemia. Whether or not such a system operates in the chick embryo is not known.

The argument against a GI hormone stimulation of CT release is two fold. First the system as outlined by Swaminathan et al (1973) has only been demonstrated in meal eating animals in which there is a discontinuous flow of food down the GI tract, and secondly the reason for the fall in CT levels from stage 20C onwards would need to be explained. Certainly, in the chicken, the presence of a crop ensures a continuous flow of food down the GI tract (until the crop is empty) and whilst the albumen and allantoic fluid are almost completely absorbed at hatching there is still a flow of yolk into the small intestine, via the yolk-stalk, and at hatching, (and most probably for one or two days before), the intestine is the major site for the absorption of the yolk contents.

A β-adrenergic pathway for the stimulation of CT secretion is well known. Experiment 10 sought to implicate such a pathway in th chick embryo. Direct measurement of CT following i.v. isoprenaline injection was shown to stimulate CT release and even more revealing was the effect of the β-blocker propranolol. A dose of 1 mg of this drug reduced the circulating levels of CT in 19 day chick embryos to below detectable levels. This suggests that the stimulus for CT secretion in the 19 day chick embryo was almost wholly β-adrenergic in nature. Whether such a β-adrenergic stimulation of CT release is via circulating adrenaline or via a neural pathway cannot be determined from the above experiment. It is of considerable interest in this respect that a neural stimulatory pathway has been implicated in avian species. However, as yet, no physiological role for such a neural stimulatory pathway has been suggested and the conditions necessary for its activation are unknown. Returning then to speculation it may be that the respiratory state of the embryo is of importance. The PCO_2 progressively increases and the PO_2 progressively falls until the time of pipping when these states are largely relieved. It would not be unreasonable to suggest that such anoxic conditions may well stimulate adrenaline release directly from the adrenal medulla or via central nervous system pathways which in turn stimulates CT release.

Whilst the above anoxic-adreanline hypothesis is attractive in that a β-adrenergic pathway has been implicated experimentally and because there is a good temporal correlation between hypoxia and CT stimulation, it is difficult to suggest a subsequent role for CT which would relieve the prevailing stimulatory conditions. In addition the failure of the CT peak to move with an artificially induced advancement or retardation of pipping casts doubt upon the ability of hypoxia to stimulate CT release. Further, the removal of CT by specific antibodies resulted in hypercalcaemia but hypoxia and hypercalcaemia are not usually associated variables although some authors have reported a direct effect of atmospheric oxygen upon the ability of UB glands to respond to a calcium challenge in vitro.

If it is postulated that CT can function in acid-base balance this may serve to clarify the situation. In such a case hypoxia (or hypercapnia) would stimulate CT release which would then increase PO_2 or decrease PCO_2, or at least attempt to. Recently a short communication was presented at the Sixth Parathyroid Conference held in July

1977 in Vancouver (to be published in 1978). This communication (Benner D.A., Lobaugh, B. and Mueller, W.J.) reported that UBX in laying hens resulted in an increase in plasma PCO_2 and bicarbonate concentration and a small decrease in plasma pH, but had no effect upon plasma total or ionic calcium. In view of our own experiments this is a most interesting observation but clearly this is a field for future investigation.

It would be appropriate at this stage to consider the functions of PTH and 1,25-$(OH)_2$-D_3 in chick embryo calcium homeostasis. Whilst PTH concentrations have not as yet been measured in embryos a role for PTH is most likely. Similarly, the embryonic kidney is capable of synthesising 1,25-$(OH)_2$-D_3 as early as day 9 of incubation (Bishop and Norman, 1975). The hormonal control of calcium transport by the C-A membrane has yet to be investigated but it is most probably that both these hormones will prove to be of importance. The middle stages of incubation of the chick embryo represent possibly the most active period of calcium metabolism. The calcification of the developing skeleton proceeds at a relatively enormous rate and the stress placed upon plasma calcium homeostasis due to the associated transfer of calcium to the skeleton from the shell and the yolk must be considerable.

The ability of CT to regulate the 1-hydroxylation is questioned and if it is functioning in calcium metabolism it is more likely to inhibit the calcium mobilising actions of PTH. Some points have already been made in this respect but further speculation is probably best witheld until changes in PTH in the chick embryo have been determined.

The establishment of a possible restrictive role for CT in the chick embryo led us to investigate two additional situations in older cockerels and pullets and in laying hens. In the latter case the results of experiment 14 clearly indicate a function of CT in the prevention of hypercalcaemia whenever egg-shell calcification is not taking place.

These results are in agreement with those of Dacke et al 1972 and support the theory put forward by Taylor that medullary bone calcium is resorbed under the influence of PTH since we would suggest that the function of CT is to inhibit such PTH resorption unless egg-shell calcification is to take place. The most likely factor regulating the plasma concentrations of these two hormones is a fluctuation in plasma ionic calcium reported by Taylor and Hertelendy (1961).

The dietary experiment reported in experiment 13 led to the conclusion that increasing the calcium content of the diet of cockerels and pullets led to hypercalcaemia and hypophosphataemia and to small increases in circulating CT. Once again it should be emphasised that the measurement of ionic calcium concentrations would have aided the interpretation of the results. However, some useful points can still be made, notably that the ability of both cockerels and pullets to adapt their rate of intestinal calcium absorption to the dietary calcium intake was confirmed and similarly confirmation of age and sex differences was obtained. It must be stated however, that the relatively small changes in plasma CT concentration induced by high calcium diets were not as striking as the morphological and histological changes in the UB gland reported by Cipera et al 1970.

Conclusions

(1) The literature has been reviewed.

(2) A radioimmunoassay specific for chicken calcitonin has been developed.

(3) A period of mild hypercalcaemia in the chick embryo correlates well with an increase in circulating CT concentration.

(4) Calcium is unlikely to be the stimulus for the release of CT, although the UB glands do respond to a calcium challenge.

(5) It is suggested that a β-adrenergic pathway for CT stimulation may be involved and that hypoxia or hypercapnia may activate this pathway.

(6) A restrictive role for CT is implicated in the chick embryo since the degree of artificially induced hypercalcaemia is reduced by CT whereas removal of circulating CT results in hypercalcaemia.

(7) No effect of salmon CT upon the plasma concentration of a number of ions, nor upon blood or extracellular volume was observed but the experimental design of this experiment did not rule out possible renal effects.

(8) CT did have transient effects upon the rate of removal from the plasma of ^{45}Ca and $^{32}PO_4$.

(9) The importance of the yolk-sac in calcium homeostasis in the chick embryo is stressed.

(10) CT may also restrict hypercalcaemia in cockerels and pullets fed high calcium diets.

(11) CT is convincingly shown to be involved in plasma calcium homeostasis during the ovulatory cycle of the laying hen. It was found in detectable levels in the plasma of hens which were not calcifying an egg-shell but not at other times.

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