THE STUDY OF SEVERAL PROTEINS SYNTHESIZED AT VARIOUS STAGES OF CHICK DEVELOPMENT

by

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ABSTRACT

A method was developed for the assay of very small quantities of radioactively-labelled peptide chains of human hemoglobin based upon the formation of complex with porcine haptoglobin, and subsequent precipitation of the complex with antiserum to the haptoglobin. Attempts to apply the method to a kinetic study of synthesis of the globin chains of embryonic chick hemoglobins in explanted chick blastodiscs were unsuccessful. Exploratory studies indicated one of the reasons for this failure is probably a relatively low affinity of the embryonic chick Hbs for porcine haptoglobin. Although the plasma of 8 week old chicks contained a haptoglobin, no evidence was obtained for the presence of this protein in the circulation of the 10 day embryo and supporting membranes.

Reports in the literature indicate that levels of aminolevulinate synthetase enzyme present in individual chick blastodiscs are sufficient for assay by highly sensitive radiochemical methods. However activity was not detectable by the most sensitive method of assay currently available and prior positive observations reflect artefacts of the assay method used.

The course of development of serotonin N-acetyltransferase activity in the chick pineal during development to 18 days post hatch has been established. Modifications of the conditions of lighting

had no appreciable effect upon the course of increase in activity. However, a diurnal cycle in levels of activity was observed with birds examined at 16 days post hatch. Preliminary studies of organ cultures of embryonic chick pineal gland indicate that the developmental increase in serotonin N-acetyltransferase activity is probably not induced solely in response to norepinephrine.

LIST OF ABBREVIATIONS AND SYMBOLS

ALA	δ aminolevulinic acid
AMP	Adenosine 5'-phosphate
antisera/2	Antisera mixed with an equal volume of borate saline
borate saline	.1M borate buffer (pH 8.4) plus 0.9% NaCl (95/5:V/V)
cpm	counts/minute
D:D	continual darkness (0:24) no hours of light 24 hours of darkness
DNA	Deoxyribonucleic acid
Hb	Hemoglobin
HbHp	Hemoglobin haptoglobin complex
HIAA	Hydroxyindole acetic acid
HIOMT	Hydroxyindole-O-methyl transferase
Hp ($\alpha\beta$)	A Hp molecule half saturated with Hb
Hp $(\alpha\beta)_2$	A Hp molecule completely saturated with Hb
L:D	Diurnal lighting e.g. (12:12) 12 hours of light and 12 hours of darkness over a 24 hour period
L:L	Continuous light (24:0) 24 hours of light no hours of darkness
MAO	Monoamine oxidase
MEL	Melatonin
MIAA	Methoxyindole acetic acid
mCi	Milli Curie

mμ	Millimicron
NAS	N acetylserotonin
NAT	Serotonin N acetyl transferase
nM	Nana mole (10^{-9} mole)
O.D.	Optical Density
POPOP	(1,4-Bis-/2-5 phenyloxazolyl/)-benzene
PPO	2,5 - diphenyloxazole
Px	Pinealectomized
SER	Serotonin
TCA	Trichloroacetic acid
TCL	Thin layer chromatography
Tris	Tris-(hydroxy methyl)-aminomethane
μCi	Micro Curie 10^{-6} Curies
μg	Microgram 10^{-6} grams
u.v.	ultra violet
αβ	A Hb dimer consisting of 1α and 1β chain
$(\alpha\beta)_2$	Hb tetramer
λ	10^{-3} ml
γ	10^{-3} mg

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Project I

Attempted development of an assay for the de novo synthesis of hemoglobin peptide chains in the chick blastodisc based upon complex formation with haptoglobin.

INTRODUCTION

The first site of hemoglobin formation in the developing chick blastodisc is the blood islands of the area vasculosa, a horse-shoe shaped region of tissue surrounding the posterior portion of the embryo proper. Heme and hemoglobin are first detected in the blood islands of individual blastodiscs at the 6-7 somite stage of development (27-33 hours of incubation). When young blastodiscs are explanted onto a suitable media prior to this stage the blood islands continue to mature and form visible quantities of Hb within 24 hours. A number of investigators have examined the effects of exogenous supplements on the formation of Hb in such explanted blastodiscs (Wilt, 1965, 1966; Levere et al., 1967; O'Brien, 1959, 1961; Hell, 1964). In particular several workers have reported the stimulation of Hb formation by supplements of δ aminolevulinic acid (ALA) (Levine and Granick, 1965; Wainwright and Wainwright, 1966, 1969). This increase has been formally demonstrated to represent de novo synthesis of Hb peptide chains.

Wainwright (1970) has developed a highly sensitive assay for low levels of Hb present in the young chick blastodisc based upon the peroxidase activity of free heme. Wainwright and Wainwright (1970) have applied this assay to the kinetic studies of the formation of functional Hb in the explanted chick blastodisc during development between the primitive streak and 12 somite stages under various

conditions. They confirmed the stimulation of Hb formation in explanted blastodiscs. However they further observed that the extent of stimulation of Hb formation by ALA was markedly dependent upon the initial stage of development of the embryo. Moreover the stimulation was transitory and was essentially complete after 9 hours of incubation. These observations support the hypothesis that the demonstrated stimulation of Hb formation by ALA in the young chick blastodisc reflects the presence of a substantial pool of free preformed globin chains prior to onset of rapid formation of heme, rather than a stimulation of de novo synthesis of globin chains.

It therefore seems important to determine the kinetics of formation of the globin chains in the young blastodisc. In theory such a study should be possible by assay of the kinetics of incorporation of radioactive amino acids in material precipitated by a highly specific antiserum directed against adult hen Hb (Wilt, 1962). However, hen Hb is a notoriously poor antigen and we have been unable to produce or purchase antiserum of reasonable titer. Nevertheless, it seemed possible that a sensitive method of assay could be developed on the basis of complex formation between hemoglobin and haptoglobin.

I have developed such an assay for the model system of human hemoglobin reacting with porcine haptoglobin. I have also observed unexpected differences between chick and human hemoglobins in their interactions with the haptoglobin. Unfortunately, these included incomplete precipitation of the chick Hb porcine Hp complex in combination with Hp antiserum.

I. BACKGROUND TO STUDY

1. Hemoglobins

(a) General Description

The hemoglobins of many species of vertebrates, including man, have been extensively studied for many years. The proteins have molecular weights of approximately 68,000 and contain four protein subunits linked to an iron containing heme group, arranged in the form of an irregular tetrahedron (Perutz, 1969). The protein subunits consist of two pairs of polypeptide chains which in the case of the major species of Hb present in normal adult animals are designated the α and β chain, and using this nomenclature the Hb tetramers can be represented as $(\alpha\beta)_2$. Other minor species of Hbs are found in adults and other distinctive molecular species are found in embryos and young animals (Braunitzer, et al., 1964; Antonini and Brunori, 1970).

The primary structure of the peptide chains of many of the vertebrate Hbs has been determined (Dayoff, 1972). Comparison of these amino acid sequences reveals substantial homologies between both (a) the various types of Hb chains found at various stages of development of a single species of animal and homologous myoglobins and (b) the adult types of Hbs found in different animal species. Indeed it has been possible to construct evolutionary trees based on these sequence homologies (Dayoff, 1972).

The secondary and tertiary structure of several molecular species of Hb has been determined by X-ray analysis (Perutz, 1969). The interior of the molecules usually contain non polar residues while at surface sites both non polar and polar residues are interchanged with little effect upon tertiary structure. Non polar forces which hold the Hb tetramer together exist mainly between unlike subunits, contacts between like chains taking little part (Perutz, 1969). As a result in mild dissociating agents (pH 4.5 or 2.5 M guanidine hydrochloride) Hb splits into 2 $\alpha\beta$ dimers. When the pH is below 4.5 or 6 M guanidine hydrochloride is used the 2 $\alpha\beta$ dimers split again into separate α and β chains. The equilibrium between Hb and its subunits can be represented:

$$(\alpha\beta)_2 \rightleftarrows 2(\alpha\beta) \rightleftarrows 2\alpha + 2\beta$$

A comparison of the structures of oxy and deoxy forms of Hb has indicated the relationship between structure and physical function.

(b) Adult Chicken Hb types

Adult chicken Hb separates into two major types (Hb A and Hb D) on standard polyacrylamide gel electrophoresis. The former accounts for 70-80% of the total and has the slower mobility, being less acidic (Bruns and Ingram, 1973; Manwell et al., 1966). Other minor Hbs (less than 1%) have been reported (Matsuda and Takei, 1963; Godet et al., 1970). As expected Hb A contains

fewer acidic amino acids (Allen, 1972; Saha, 1964; Moss and Thompson, 1963). The β chains of Hb A and Hb D are quite similar in amino acid content and yield identical peptide maps (Moss and Thompson, 1969). The αA and αD chains however are quite different. Sequence work on Hb A has been completed (Matsuda et al., 1971; Matsuda et al., 1973). The αA chain contains 141 amino acids, the βA 146. Although each chain contains the same number of amino acids as their human counterpart, they differ from them in primary sequence by 35 and 45 amino acids respectively.

(c) Fetal Chicken Hb Types

Chick fetal Hbs have not been as extensively studied because of the difficulty in obtaining large amounts of material. Two major Hb types have been reported (Fraser, 1964; Manwell et al., 1966; Bruno et al., 1973) consisting of 70% Hb P and 20% Hb E. These embryonic Hbs may have a common chain but all chains are different from the adult Hbs (Manwell et al., 1963; Hashimoto and Wilt, 1966). Several minor components (less than 1%) have been reported (Godet et al., 1971; Hashimoto and Wilt., 1966).

2. Erythropoiesis in the Developing Chick Embryo

Hemoglobin biosynthesis in the chick embryo has been extensively studied (Lucas and Jamroz, 1961; Lemez, 1964; Wilt, 1967; Bruns and Ingram, 1973).

On the yolk of the unincubated egg is an inverted saucer like sheet of 60,000 cells. Settle (1954) and Spratt and Haas (1960) by explantation and tracing of cell movement by carbon marking showed that cells located near the marginal zone later carry on hemoglobin synthesis. During the first 18 to 20 hours of incubation a complex set of morphological movements occurs resulting in a three layered mass of cells (mesodermal subdivided between an ectodermal and an endodermal layers) with the future neural axis of the embryo along the midline. Descendants of the original marginal zone of cells now lie in the middle mesodermal layer lateral to what will become the posterior portion of the embryo. The mesoderm layer condenses at about the headfold stage (22-24 hrs. of incubation) to form small compact cell aggregates, the blood islands in which the red blood cells of the primitive cell line are first observed. The cells of the surface of the blood islands flatten to form the endothelia of the blood vessels.

Heme (Levine and Granick, 1965) and hemoglobin (Wilt, 1967) can first be detected in these blood islands at the 6-7 somite stage of the blastodisc (27-33 hrs. incubation). Also at this time an antigenic component typical of fetal avian Hb can be demonstrated by immunodiffusion techniques (Wilt, 1962). Antiserum specific for fetal Hb is also capable of precipitating material which has incorporated (^{59}Fe) and (^{3}H)-leucine. All these observations demonstrate onset of a rapid synthesis of Hb occurring at the 6-7 somite stage.

Wainwright and Wainwright (1970) using a sensitive assay for acid extractable heme to study the kinetics of Hb formation in the chick embryo also showed a rise in the rate of Hb synthesis of the 6-7 somite stage (Figure 1). Furthermore, embryos as early as the primitive streak stage contained small but detectable amounts of acid extractable heme. The rate of formation of functional Hb declined between the 2 and 3 somite stages of development. This is presumptive evidence of the possible presence of an embryonic Hb prior to the 3 somite stage. These findings are in accord with Wilt (1962) who demonstrated the presence of material at the primitive streak stage which reacted with specific anti-chick Hb antiserum but was neither fetal nor adult chick Hb.

This rapid rise in Hb production appears to be regulated by several factors including the supply of ALA (Figure 2). Supplements of ALA added to explanted chick blastodiscs lead to stimulation of Hb production (Levine and Granick, 1965; Wilt, 1965, 1967; Wainwright and Wainwright, 1966, 1970). Synthesis of this key intermediate by mitochrondrial ALA synthetase is the rate limiting step in the formation of heme prosthetic groups (Granick and Levine, 1964; Levine and Granick, 1965, 1967).

Coincident with the rapid Hb synthesis at the 6-7 somite stage is the appearance of the first primitive erythroid cells. These cells exist in the embryonic circulation as a cohort of cells, which all tend to be at the same maturation stage at the same time.

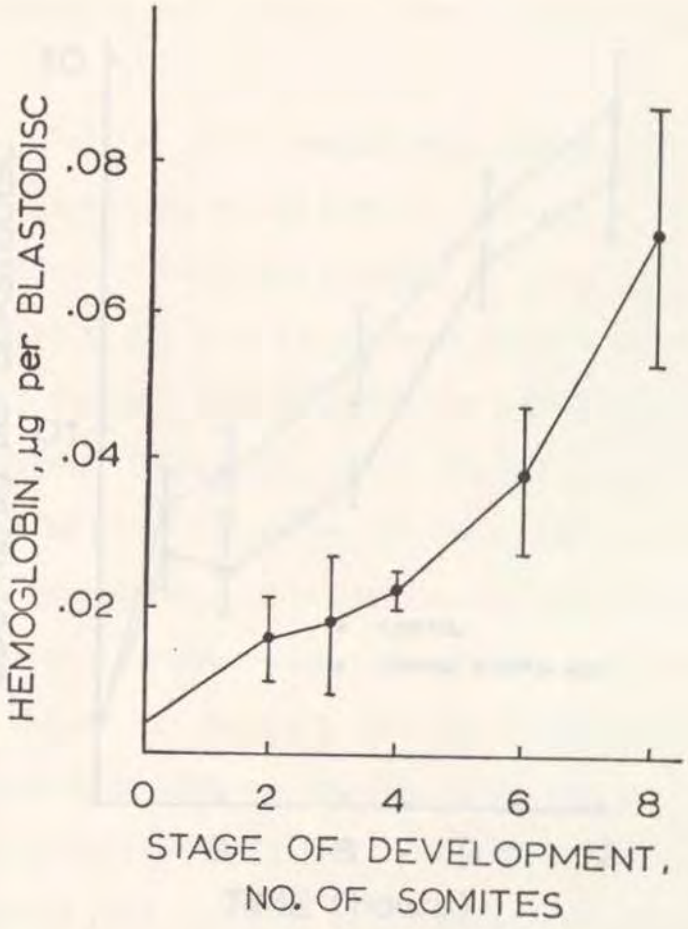


Figure 1: Hemoglobin synthesis in ovo. Hemoglobin was assayed in pools of blastodiscs collected immediately after dissection. Approximately 5 hours are required beyond the stage of the definite primitive streak (0 somites) for organization of the first pair of somite blocks and a further hour for each additional pair of somites. The bars indicate one standard deviation. From Wainwright and Wainwright (1969).

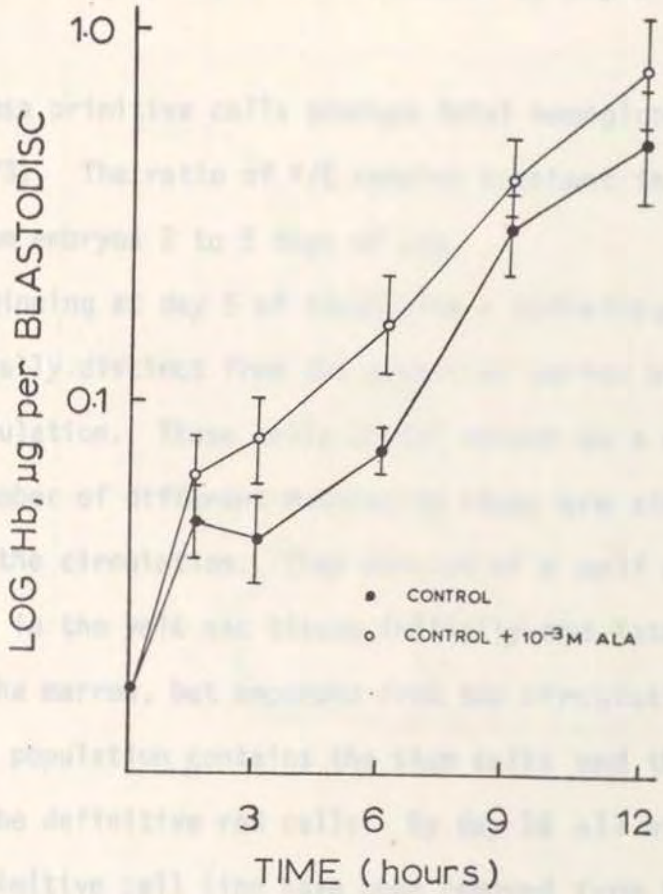


Figure 2: Hemoglobin synthesis in vitro; effect of supplements of ALA. Blastodiscs were explanted onto rich media at the 3 somite stage and the course of Hb formation was followed. The bars indicate one standard deviation. From Wainwright and Wainwright (1969).

They stop dividing by day 5 and are mature by day 9 (Campbell et al., 1971).

These primitive cells produce fetal hemoglobins (Bruns and Ingram, 1973). The ratio of P/E remains constant in erythroid lysates from embryos 2 to 5 days of age.

Beginning at day 5 of incubation a definitive cell line morphologically distinct from the primitive series begins to appear in the circulation. These cells do not mature as a cohort but rather a number of different maturation steps are simultaneously present in the circulation. They consist of a self renewing population sequestered in the yolk sac tissue initially and later by day 14 are formed in the marrow, but separate from the circulation. The sequestered population contains the stem cells and the most immature stages of the definitive red cells. By day 16 all of the cells from the primitive cell line have been removed from the circulation and only cells of the definitive type remain (Figure 3).

Upon appearance of the definitive erythroid cells two new Hb components are observed on polyacrylamide gel electrophoresis (Bruns et al., 1973). These new Hbs were shown to be identical to the adult chick Hbs A and D by both electrophoretic and immunological techniques. The amounts of Hb A and D increase while Hbs P and E decrease as the embryo matures until day 16 when no Hb P can be demonstrated either electrophoretically or iimmunologically in erythroid cell lysates. This loss of Hb P ccorresponds well with the

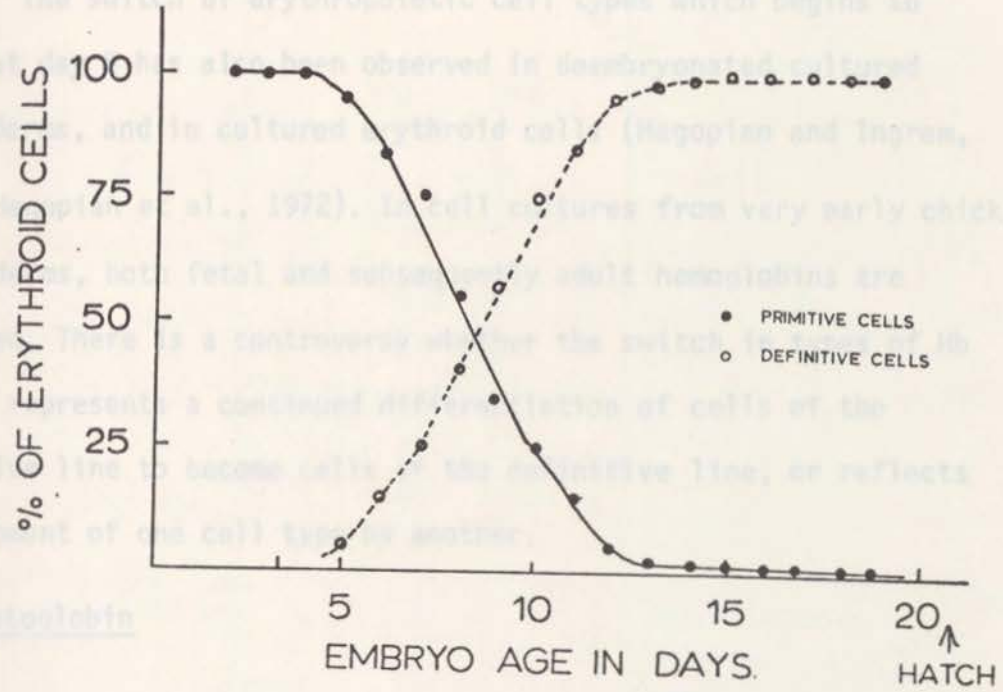


Figure 3: Percentage of primitive and definitive erthyroid cells from 2 to 19 day old chick embryos. From Bruns et al. (1973).

removal of the primitive cell line from the circulation.

The switch of erythropoietic cell types which begins to occur at day 5 has also been observed in deembryonated cultured blastoderms, and in cultured erythroid cells (Hagopian and Ingram, I97I; Hagopian et al., I972). In cell cultures from very early chick blastoderms, both fetal and subsequently adult hemoglobins are produced. There is a controversy whether the switch in types of Hb formed represents a continued differentiation of cells of the primitive line to become cells of the definitive line, or reflects replacement of one cell type by another.

3. Haptoglobin

This α_2 glycoprotein has been the subject of several reviews (Jayle and Moretti, 1962; Laurell and Gronwall, 1962; Hamaguchi, 1969; Sutton, 1970).

Human haptoglobins consist of three genetically determined phenotypes, Hp 1-1, Hp 2-1 and Hp 2-2, (Smithies and Walker, 1956). Hp 1-1 (Malchy and Dixon, 1973) is a single homogeneous protein (MW 98,000) consisting of 2 light and 2 heavy chains joined by disulfide bridges (Figure 4). There are no disulfides between H chains. After extensive reduction by mercaptoethanol in 8 M urea the refolding of Hp is spontaneous and up to 80% native Hp can be recovered (Bernini, 1970). The amino acid sequence of the L chain

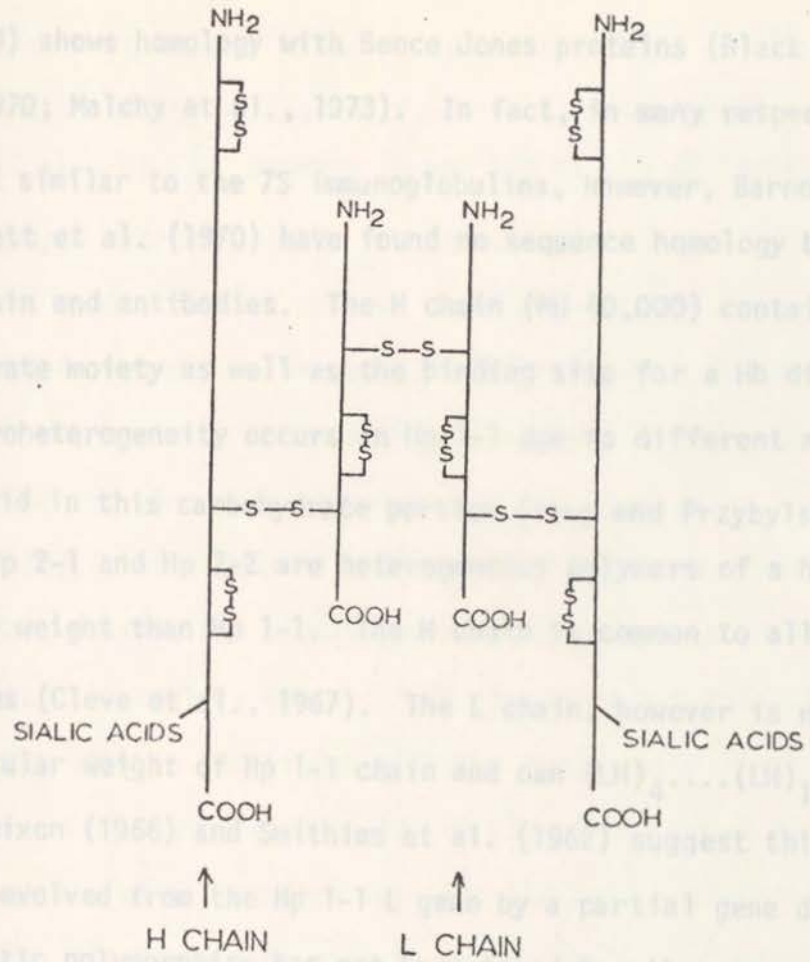


Figure 4: The human Hp molecule. A model based on the work of Shim and Bearn (1964) and Maddy et al. (1973). The sialic acids are linked to the H chains and there are no direct disulfide linkages between H chains.

(MW 9,000) shows homology with Bence Jones proteins (Black and Dixon, 1970; Malchy et al., 1973). In fact, in many respects Hp 1-1 is similar to the 7S immunoglobulins, however, Barnett et al, (1972) and Barnett et al. (1970) have found no sequence homology between the H chain and antibodies. The H chain (MW 40,000) contains the carbohydrate moiety as well as the binding site for a Hb dimer. Some microheterogeneity occurs in Hp 1-1 due to different amounts of sialic acid in this carbohydrate portion (Yang and Przybylska, 1973).

Hp 2-1 and Hp 2-2 are heterogeneous polymers of a higher molecular weight than Hp 1-1. The H chain is common to all Hp phenotypes (Cleve et al., 1967). The L chain, however is nearly double the molecular weight of Hp 1-1 chain and can $(LH)_4$....$(LH)_{10}$ (Fuller et al., 1972). Dixon (1966) and Smithies et al. (1962) suggest this new L chain may have evolved from the Hp 1-1 L gene by a partial gene duplication. This genetic polymorphism has not been found in other mammals and they contain a Hp phenotype similar to Hp 1-1 (Jayle and Moretti, 1962; Cheftel et al., 1971). Pig Hp $(LH)_2$ has a molecular weight of about 95,000 (Fraser and Smith, 1970; Shim et al., 1971) and H chains are not linked by disulfides (Lockhart and Smith, 1971).

Although Hp has been found in other vertebrates such as the frog, lamprey and duck (Sasazuki, 1971), there are conflicting reports as to whether or not the domestic fowl contains this protein (Darcel and Bide, 1969; Riou et al., 1962).

The fact that Hp binds Hb specifically has led to an understanding of its biological function (Allison and Rees, 1957; Murray et al., 1961). Between 70 and 90% of old erythrocytes are destroyed extravascularly by the reticuloendothelial system (Keene and Jandl, 1965). However some lyse intravascularly releasing their Hb into the plasma where it is bound by Hp. In hemolytic states in which plasma Hp is depleted, circulating free Hb readily transverses the glomeruli of the kidney and appears in the urine (Bunn, 1972). This leads to kidney damage (Pintera, 1968) as well as the loss of iron and protein from the body stores. HbHp complex, in contrast, is rapidly removed from the blood and catabolized in the liver. This is consistent with the report of a liver enzyme heme α methenyl oxgenase (Nakijima et al., 1963) which changes the hemoglobin of the complex specifically into a precursor of biliverdin. In summary Hp acts in the catabolism of plasma Hb resulting in protection of the kidney and conservation of both iron and protein amino acids for the body stores.

4. HbHp Complex

The binding of Hb to Hp is rapid (Nagel and Gibson, 1967), occurs over a pH range of 4.6 to 11.0 and is essentially irreversible (Adams, 1969). Although the binding is strong no covalent linkages are involved since Hp contains no free sulfhydryl groups and treatment

of Hb wth iodœcetamide or parachloromercuribenzoate has no effect on complex formation (Bunn, 1967). Probably hydrogen, hydrophobic and electrostatic forces account for the complexes' stability (Robert et al., 1956).

Hb is modified on binding with Hp such that it shows properties similar to free α and β chains (Brunori et al., 1966) rather than intact tetramers. It binds ligands with high affinity, shows no co-operation between subunits (n = 1) and has no Bohr effect (Nagel et al., 1965; Nagel and Gibson, 1966). This absence of co-operativity is further demonstrated by complexes containing a mixed hybrid Hb $(\alpha\ NO\ \beta)_2$ in which the α chains have been reacted with nitric oxide and the β chains were unliganded (Bannai and Sugita, 1973). The absorption spectrum of the tetramer $(\alpha\ NO\ \beta)_2$ shows a reduced molar coefficient compared with the averaged value for free α NO and B chains. However, the complex, in contrast has a high molar coefficient.

Besides a difference in ligand binding properties the environment of the heme groups in the complex is altered. We have already noted that HbHp is a specific substrate for the heme enzyme α methenyl oxygenase. Exchange of heme which occurs between Hb and albumin is blocked (Bunn and Jandl, 1968). The complex exhibits an increase in peroxidase activity (Jayle et al., 1962; Smith and Beck, 1967) as the Hb is protected by Hp from the acidic denaturation conditions used in the assay (MacKinen et al., 1972).

Even though significant changes in Hb tertiary and quaternary structure occur after combination with Hp, there are no alterations in secondary structure. The Hb α helical content and the amount of Hp structure remain unchanged (Waks et al., 1971).

5. Antigen-Antibody Reactions, the HbHp Reactions and a Comparison

Human Hb injected into a rabbit elicits the formation of antibodies (Noble et al., 1969) directed against this foreign protein. Malchy and Dixon (1970) suggested that Hp acts functionally as a Hb antibody too. Since Hb is normally present in the red blood cells when it is released into the plasma by hemolysis it can be considered as a protein foreign to that particular compartment of the body. Both Hp and anti Hb antibody show structural similarities as already described and both bind specifically to Hb. Nevertheless, there are distinct differences between their modes of action (Sasazuki, 1971; Cohen-Dix et al., 1973). In particular the HbHp complexes do not show complement fixation, a characteristic of most antigen antibody reactions (Sasazuki, 1971; Malchy and Dixon, 1970). A comparison between these two proteins has led to a better understanding of the HbHp reaction by clarifying and confirming earlier reports.

The anti-Hb antibodies attach to 12-16 sites on the external surface of the Hb tetramer involving regions on both α and β chains (Reichlin, 1972; Noble et al., 1969). These antibodies react equally well with both liganded and unliganded Hb molecules. Nevertheless, the antibody is highly species specific showing no affinity for other mammalian Hbs. In fact proteins identical to this antigen except for a single amino acid replacement may not bind antibody (Nisonoff et al., 1970; Reichlin, 1972). Because of this species specificity the antigenic sites are most likely in regions of the Hb surface which vary greatly in evolution.

As antibodies are bivalent one molecule reacts with two different molecules of Hb leading to lattice formation and precipitation. The association between antigen and antibodies is readily reversible. Exchange occurs between bound and unbound antigen and excess antigen will solublize antigen-antibody complex. Despite a low binding affinity these antibodies effectively remove antigen from solution by taking advantage of multiple determinants resulting in eventual precipitation.

In contrast a molecule of Hp combines with a single Hb molecule. Lattice formation cannot occur and there is no precipitation. It may be recalled that Hp contains two Hb binding sites. Laurell (1959) noted that two types of HbHp complexes are formed when Hp is present in excess. One contains a half molecule of Hb and the other a whole

molecule. Since then Hamaguchi (1967), Kawamura et al. (1972) and Peacock et al. (1971) have shown that the half molecule of Hb is an $\alpha\beta$ dimer. Each binding site is independent of the other. The relative amounts of Hp, Hp$\alpha\beta$ and Hp $(\alpha\beta)_2$ present after complex formation are given by the terms p^2, 2pq, and q^2 respectively in the expansion $(p + q)^2$ where $p = q-1$ and q is the fraction of Hp saturated with Hb.

Nagel and Gibson (1967) and Chiancone et al. (1968) indicate that free α chains bind half of the Hp sites whereas free β chains show no affinity at all. However, β chains bind readily to Hp half saturated with α chains. These β chains are not simply binding to the attached α chains as human Hb dimers will displace mouse α chains from a pig Hp mouse α chain saturated complex (Boyd et al., 1971). Therefore once α chains bind, β specific sites are created. These results indicate a reaction scheme for Hb and Hp (Figure 5).

Hp binds specifically to all mammalian Hbs and the binding site is probably on a portion of the Hb which has been conserved. Such an area occurs at the contact regions of the Hb dimer which is located in the interior of the tetramer. The amino acids involved in this contact region are identical for all mammalian Hbs (Perutz, 1969; Dayoff, 1972). Several other observations support this view that the binding site for Hp is not on the external surface of the Hb tetramer. Hb whose lysyl groups are treated with ethylacetimidate shows unimpaired Hp binding even though most of the lysyl groups are on the

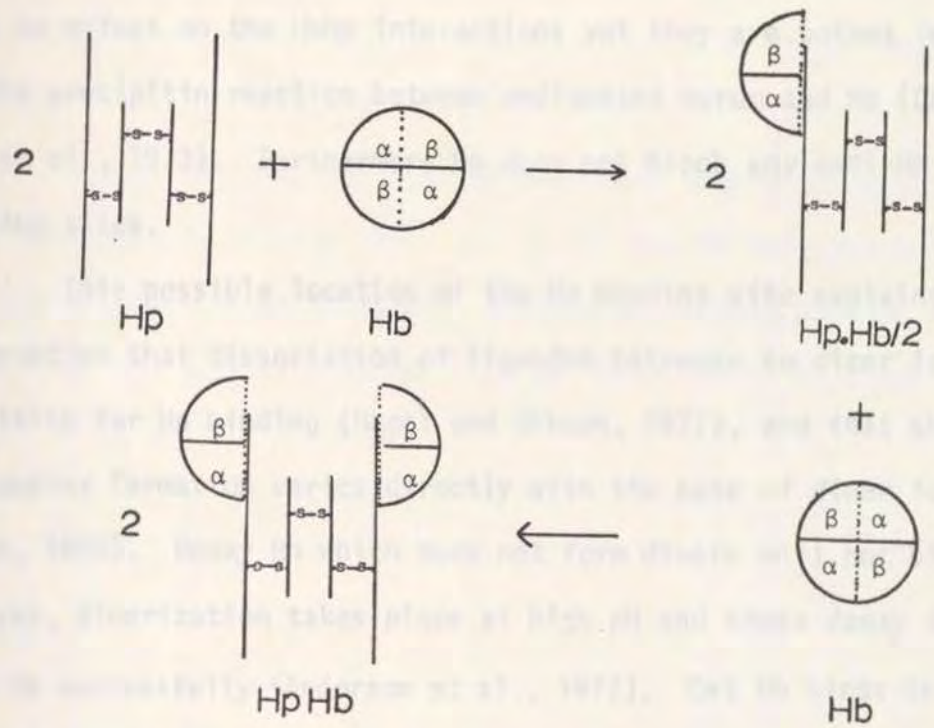


Figure 5: The reaction of Hb with Hp; a diagramatic representation. The Hp molecule contains 2H and 2L chains linked by disulfide bonds. When Hp is in excess over Hb an unsaturated HbHp complex may be formed. However when Hb is in excess each Hp molecule binds to two Hb dimers. The work of Lockhart et al. (1971) and Cohen-Dix et al. (1973) suggests that the Hp binding sites are in the interior surfaces of the Hb tetramer as illustrated.

Pavlicek and Jaenicke (1971) found similar results using bovine Hb. When human Hb is reacted with human Hp the complexes so formed are all stable and independent of the initial molar ratios of Hb or Hp (MacKinen et al., 1972).

These differences in binding are not entirely unexpected as the variation in primary structure of the Hbs is considerable. Recently Cohen-Dix et al. (1973) have reported that several non-mammalian Hbs such as carp and frog Hbs bind weakly to human Hp.

II. METHODS

1. Preparation of Hb.

Human and 19 day chick embryo Hbs were prepared essentially by the method of Drabkin (1946). In the case of 5 day chick Hb the embryos were bled into a dish of ice cold 0.9% saline and embryos and pieces of yolk were removed by passing the cells through cheese cloth. After each centrifugation the red cells were resuspended in the saline and percolated through a small column containing glass wool until all the remaining yolk material was removed. The cells were lysed in H_2O and toluene and centrifuged. The isolated Hb was stored frozen either in the carbon monoxy or cyanomet form.

2. Preparation of Pig Hp.

Pig Hp was prepared by the method of Connell and Shaw (1961) as modified by Fraser and Smith (1971). After desalting the purified Hp was freeze dried and stored at 0^{o} C until use. The Hp was over 95% pure as it gave a single band on polyacrylamide gel electrophoresis. No impurities remained at the position of the Hp band when an excess of Hb was added.

3. Preparation of antiserum.

Six New Zealand rabbits were immunized against Hp as described by Campbell et al. (1963). Each rabbit was injected

three times over a period of a month with 2 mg. of Hp in Freud's Adjuvant. At the end of this time the blood was collected and allowed to clot. The serum was stored in the fridge in 1:10000 merthiolate.

4. Purification of antiserum.

The rabbit antisera to Hp was purified as described by Campbell et al. (1963). To a volume of antisera was added half a volume of saturated $(NH_4)_2SO_4$ and the pH was adjusted to 7.8 with 2N NaOH. The solution was stirred for 3 hrs. and then centrifuged at room temperature. The precipitate was dissolved in 0.9% saline and reprecipitated twice more. $(NH_4)_2SO_4$ was removed from the purified antisera by G-25 Sephadex column chromatography in borate saline.

5. Antigen-antibody Precipitation Studies.

To a known amount of HbHp complex was added an equal volume of antisera/2. The mixture was incubated at 37°C for 30 minutes and then left in the cold room for several hours. For the precipitin curves and in some of the precipitation studies the mixture was left in the cold room for three days, the precipitate being resuspended each day. The mixture was then centrifuged and the supernatants analysed for the presence of HbHp. Antisera/2 was prepared by adding an equal volume of borate saline to the antisera. Borate saline consisted of 0.1 M borate pH 8.4 and 0.9% saline (5:95; V/V).

volumes and O.D. at 418nm of the two pooled samples were determined.

$$\text{per cent Hb bound by Hp} = \frac{\text{Vol. of HbHp peak x O.D.}}{\text{Vol HbHp peak x O.D. + Vol Hb peak x O.D.}}$$

The other method involved calculating the area of each peak either from a graph or by tracing each peak on weighing paper and weighing them.

$$\text{per cent Hb bound} = \frac{\text{area of HbHp peak}}{\text{area HbHp peak + area Hb peak}}$$

The amount of Hp present in 100 ml. of sample was then calculated from the following formula assuming Hp has a molecular weight of 95,000

$$\text{mg Hp in 100 ml plasma} = \frac{100}{0.5\ (\text{vol sample to column})} \times \text{Hb (mg Hb added to sample)} \times \text{\% Hb bound to Hp} \times \frac{95000}{68000}$$

9. Determination of Radioactivity.

Samples were dried on paper discs and placed in scintillation vials to which was added 10 ml. of a scintillation cocktail made up of 5 gm PPO (2-5 Diphenyloxazole), 0.3 gm. POPOP (1,4-Bis-/2-5 phenyloxazolyl/)- benzene per liter of toluene.

Liquid samples were counted in Brays solution consisting of (5 gms PPO, 100 gms Napthalene)/liter of dioxane. The liquid was always less than 0.5 ml to which was added 1 ml of 95% ethanol and 10 ml of the Brays solution.

Aquasol was also used for counting liquid samples. Approximately 500 λ of material to be counted was added to 5 ml of aquasol.

Samples were counted using a Beckman liquid scintillation system.

10. Polyacrylamide Gel Electrophoresis.

Electrophoresis at pH 8.3 was performed according to the method of Davis (1964). Gels were stained with Coomassie Brilliant Blue for protein (Fairbanks et al., 1971) or by peroxidase activity for Hb or HbHp (Ferris et al., 1966). Electrophoresis at pH 4.5 in urea gels was performed according to the method of Reisfield et al. (1962) and Fambrough et al. (1968).

11. Cellulose Acetate Electrophoresis.

Electrophoresis at pH 7.0 was performed according to the method of Pantlitschko and Weippl (1968). Strips were scanned on a Joyce Loebb Chromoscan densitometer.

12. Column Chromatography.

The separation of HbHp complex from Hb was performed

using 1.5 x 30 or 2 x 23 cm columns of G-100 Sephadex (Lionetti et al., 1964). Samples were eluted either in 0.9% NaCl or in borate saline pH 8.4. The flow rate was 20 mls/hr.

The plasma profiles were performed using G-200 Sephadex and a column (2.3 x 33 cm). The eluting buffer was borate saline and the flow rate 40 ml/hr. All column runs were performed at room temperature 24-26^{o} C.

G-25 Sephadex columns (2.3 x 33 cm.) were used in the preparation of met Hb and the separation of unreacted radioactive label from Hb solutions. The eluting solvent was either 0.9% NaCl or borate saline (pH 8.4).

Exchange experiments between different types of Hb and pig Hp were performed on a Sephadex G-100 column (1.5 x 30) equilibrated in borate saline (pH 8.4) at a flow rate of 30 ml/hr.

A G-200 Sephadex column (2.5 x 100) equilibrated in 0.1 m Tris pH 7.4 was used in the purification of Hp. The flow rate was 12 ml/hr and the column was maintained in the cold room at 0^{o} C.

13. Ultrafiltration.

Concentration of protein solutions was performed using a UM-10 ultrafiltration membrane. Separation of various sized proteins was performed using XM-50 and XM-100 membranes as described by the manufacturers.

14. Materials

Radiochemicals and Aquasol were obtained from New England Nuclear Corporation.

PPO and POPOP were a product of Kent Laboratories Ltd.

Purified agar from Difco Laboratories was used in the immunodiffusion studies.

Sepraphore III cellulose acetate electrophoresis strips were a product of Gilman Instrument Company.

For the DEAE-cellulose isolation of Hp procedure, Whatmann preswollen microgranular DE52 cellulose was used.

All other chemicals were reagent grade.

III. RESULTS

1. Rationale and Control of Purity of Reagents.

Two approaches to an assay for Hb based upon complex formation with Hp were envisaged provided that the Hb bound successfully to porcine Hp.

The first takes account of the large difference in molecular weight between free Hb and the HbHp complex. It was expected that passage of an homogenate of chick blastodiscs through appropriate ultrafilters or columns of Sephadex would remove all proteins of molecular weight substantially greater than that of Hb (68,000). Addition of Hp would result in formation of HbHp complex which could then be recovered as the only chick protein in the fraction of molecular weight in excess of 100,000, by repetition of the original resolution procedure. Application of this method would require that no chick Hp be formed in the blastodiscs at the stages of development used.

In the second approach it was proposed to precipitate the complex formed with Hp by means of a specific antiserum directed against the latter. Successful application of this approach is predicated on both the absence of any chick Hp from embryos and on the possibility of isolating preparations of virtually pure Hp. Ideally it would be preferable to use Hp obtained from the same

species as the Hb. However, at the time this study commenced it was even unclear whether haptoglobins are present in chick serum and methods for isolation of homogeneous preparations of possible chick Hps have yet to be developed. Moreover it was (and apparently still is (Cohen-Dix et al., 1973)) widely anticipated that interaction between avian Hbs and mammalian Hps would be entirely analagous with that between mammalian Hbs and Hps. Therefore it was proposed to use preparations of porcine Hp, for which purification procedures were already available. The Hp preparations used in this study gave a single band on polyacrylamide gel electrophoresis and a single peak on the ultracentrifuge. When the Hp was complexed with human Hb the complex moved more slowly in the gel and no protein impurity remained in the free Hp position.

Analysis of the various preparations of Hb used by polyacrylamide gel electrophoresis showed the presence of several components (Figure 6) each of which stained well for the presence of both heme and protein. Human Hb is more acidic and travels faster in the gel.

As the embryo increases in age the slowest moving Hb band disappears whereas the middle and fastest bands become darker. (Figure 6, 1 2-5). This is in accord with the work of Bruns and Ingram (1973) and suggests that with 5 and 6 day embryos we are isolating

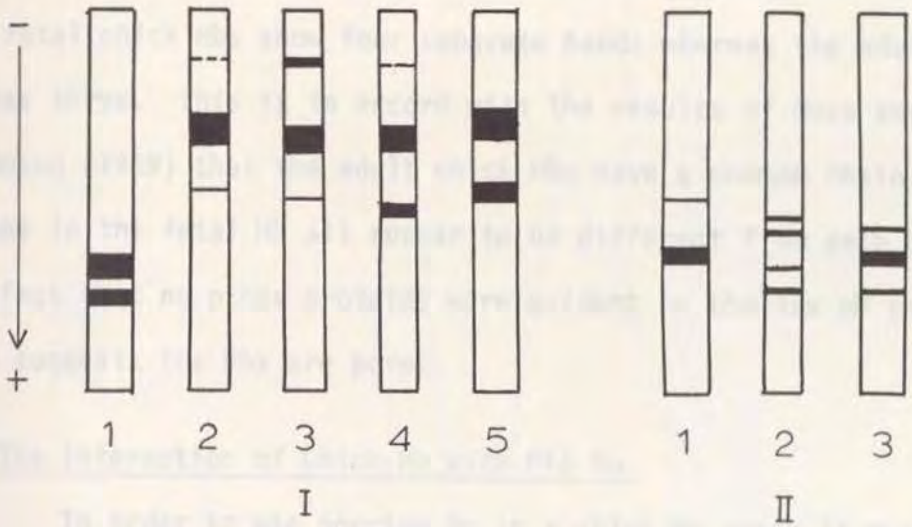


Figure 6: Polyacrylamide gel electrophoresis patterns of several Hb samples. After electrophoresis (pH 8.3; Davis, 1964) samples in group I were stained for peroxidase activity (Ferris et al. 1966). Group II samples were subjected to electrophoresis at pH 4.5 in 8 M urea according to the method of Reisfield et al. (1962) and subsequently stained with Coomassie Brilliant Blue.

I
1. Human Hb.
2. Chick Hb from a 5 day embryo.
3. Chick Hb from a 6 day embryo.
4. Chick Hb from a 7 day embryo.
5. Chick Hb from a 19 day embryo.

II
1. Human Hb.
2. Chick Hb from a 5 day embryo.
3. Chick Hb from a 19 day embryo.

the fetal Hbs whereas at day 19 the adult type is prevalent. Low pH gel runs in 8 M urea separate human Hb into free α and β chains. The fetal chick Hbs show four separate bands whereas the adult Hb has three. This is in accord with the results of Moss and Thompson (1969) that the adult chick Hbs have a common chain. The chains in the fetal Hb all appear to be different from each other. The fact that no other proteins were evident in the low pH gels also suggests the Hbs are pure.

2. The Interaction of Chick Hb with Pig Hp.

In order to use porcine Hp in a chick Hb assay it must first be shown to be able to complex with such Hb.

The binding of chick Hb to pig Hp was measured by three techniques. Sephadex G-100 column chromatography (Figure 7) of a solution of pig Hp and 5 day embryonic chick Hb gave two Hb containing fractions. The peak emerging initially from the column was the HbHp complex whereas the second peak was free Hb. Both 19 day embryonic chick Hb and human Hb gave similar results when added to Hp.

At pH 7.0 chick Hb gave a single band on cellulose acetate electrophoresis. After addition of pig plasma however two bands were present, one complex, the other free Hb (Figure 8a). For comparison the pig plasma plus human Hb scan is added as a reference (8b).

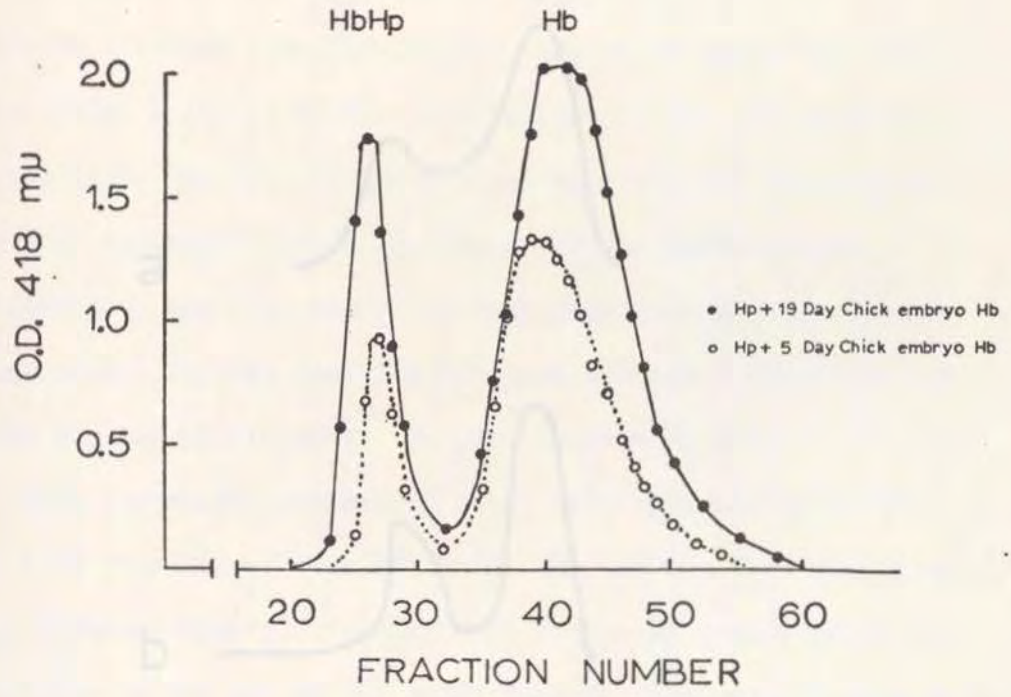


Figure 7: Sephadex G-100 column chromatography of pig Hp plus chick Hb samples. An excess of Hb was added to purified porcine Hp and chromatographed on a column 1.5 x 30 cm equilibrated in 0.9% NaCl.

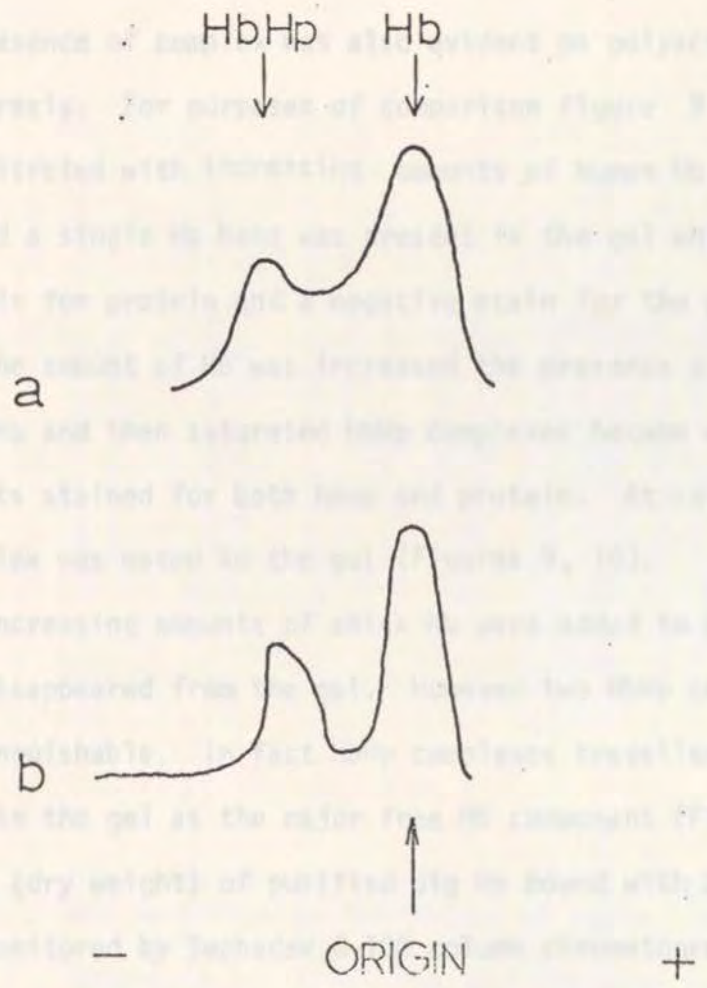


Figure 8: Cellulose acetate electrophoresis patterns of samples of pig plasma and Hb. Electrophoresis at pH 7.0 in .05 M phosphate buffer was performed according to the method of Pantlitschko and Weippl (1968) at 2.5 mA/strip for a time of 30 minutes.

(a) pig plasma + chick Hb.
(b) pig plasma + human Hb.

The presence of complex was also evident on polyacrylamide gel electrophoresis. For purposes of comparison figure 9 shows pig Hp titrated with increasing amounts of human Hb. When no Hb was added a single Hp band was present in the gel which gave a positive stain for protein and a negative stain for the presence of heme. As the amount of Hb was increased the presence of unsaturated HbHp and then saturated HbHp complexes became evident. These components stained for both heme and protein. At saturation only HbHp complex was noted in the gel (Figures 9, 10).

When increasing amounts of chick Hb were added to Hp the free Hp band disappeared from the gel. However two HbHp complexes were not distinguishable. In fact HbHp complexes travelled in the same position in the gel as the major free Hb component (Figures 11 & 12).

One mg (dry weight) of purified pig Hp bound with 0.7 mg. of chick Hb (monitored by Sephadex G-100 column chromatography) suggesting they combined in approximately an equimolar ratio. Similar results were found by the polyacrylamide gel studies.

3. Presence of Hp in Adult Chick Serum and its Absence from Serum of Young Embryos.

Direct examination for the presence of Hp in chick blastodiscs at the primitive streak to 8 somite stages of development poses formidable problems. However it was argued that if no Hp was present in the sera of older embryos or adult birds it was probably also absent from that of young blastodiscs of special interest. Therefore experiments

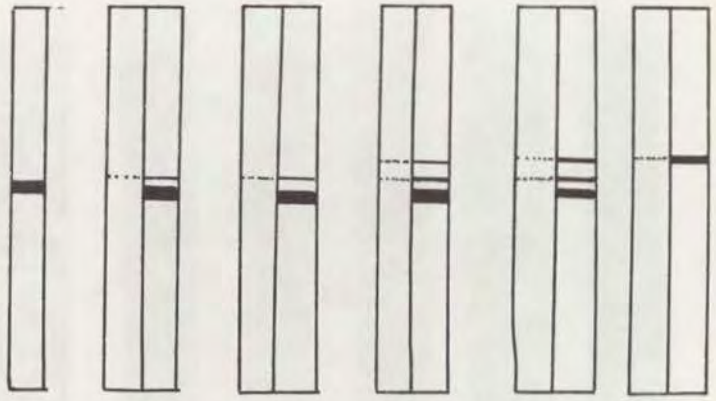


Figure 9: Titration of pig Hp with human Hb. Electrophoresis was performed by the method of Davis (1964). The odd numbered gels were stained for protein with Coomassie Brilliant Blue and the even numbered were stained for peroxidase activity. The ratio Hb to Hp in the titration series is as indicated.

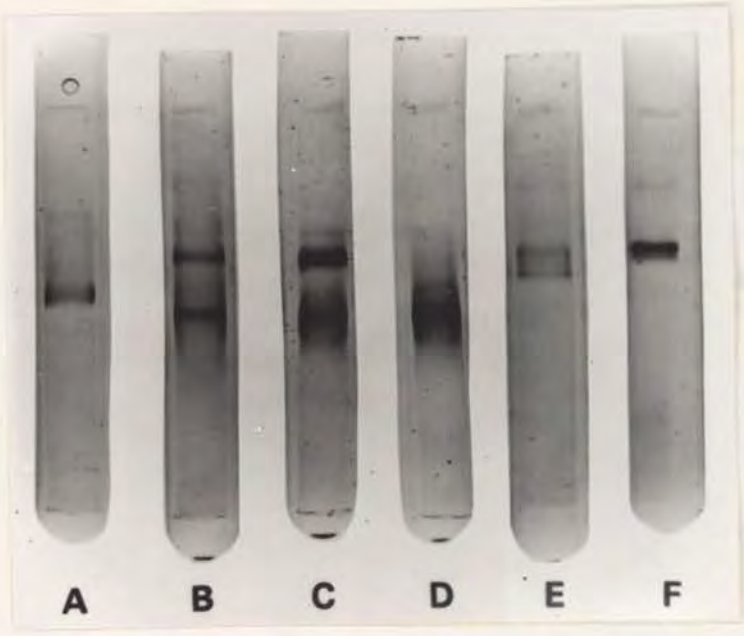
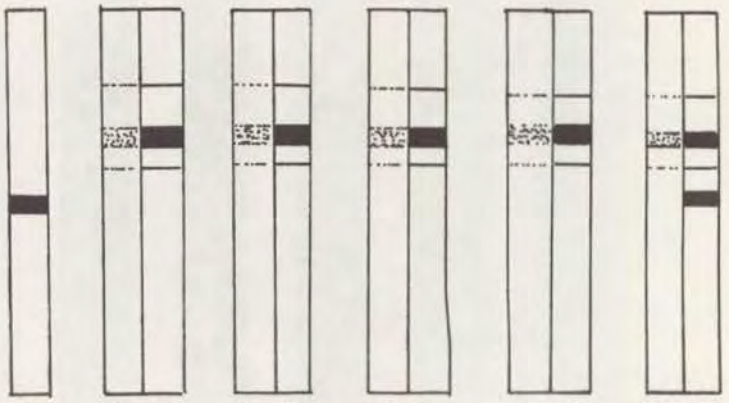


Figure 10: Polyacrylamide gel electrophoresis patterns of samples containing pig Hp and human Hb. Electrophoresis was performed at pH 8.3 (Davis, 1964). Gels C and F were stained for peroxidase activity all others were stained for protein with Coomassie Brilliant Blue.

A. Pig Hp.
B. Pig Hp plus an excess of human Hb.
C. A repeat of B stained for peroxidase activity.
D. Human Hb.
E. Pig Hp plus an unsaturating amount of human Hb.
F. A repeat of E stained for peroxidase activity.



Hb/Hp	0/1	2.6/0	5.2/1	2.6/1	1.3/1	.66/1

Figure 11: Titration of pig Hp with 5 day embryonic chick Hb. Electrophoresis was performed according to the method of Davis (1964). Odd numbered gels were stained for protein with Coomassie Brilliant Blue and even numbered for peroxidase activity. The ratio of Hb to Hp in the titration series is indicated.

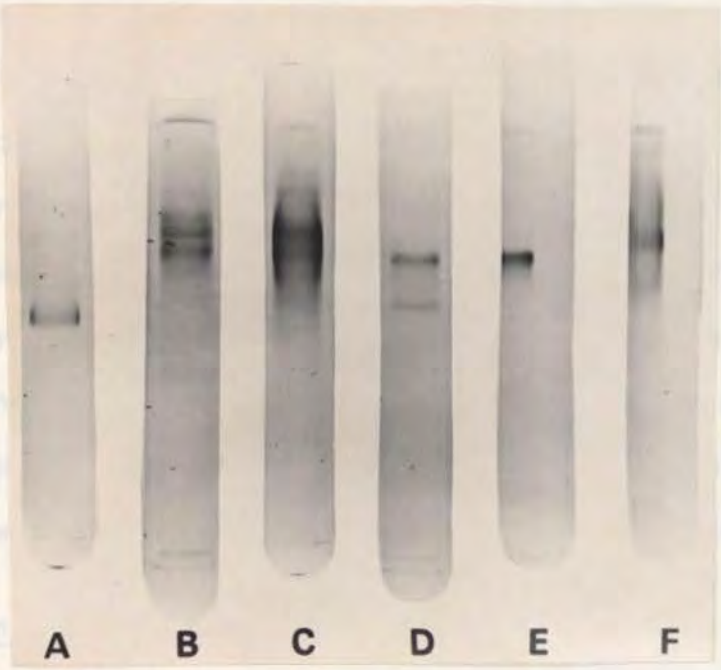


Figure 12: Polyacrylamide gel electrophoresis patterns of samples containing pig Hp and 5 day embryonic chick Hb. Electrophoresis was performed at pH 8.5 (Davis, 1964). Except where noted, all gels were stained for protein with Coomassie Brilliant Blue.

A. Pig Hp.
B. Pig Hp and an excess of chick Hb.
C. B stained for peroxidase activity.
D. Pig Hp and unsaturating amount of chick Hb.
E. D stained for peroxidase activity.
F. Chick Hb alone.

were carried out to determine whether chick plasma contains Hp.

When adult chick Hb was added to 8 week old hen serum the presence of Hp was evident as monitored by column chromatography (Fig. 13) and cellulose acetate electrophoresis (Fig. 14a). The amount of Hp present was 113 mg. per 100 ml. serum.

No Hp was present in either 21 or 10 day chick embryo plasma as monitored by cellulose acetate electrophoresis (Fig. 14b, c). The shoulder which occurs in the scan of the 21 day embryo and chick Hb samples is possibly due to the initial intense yellow color of the plasma as well as a smearing of the sample on application.

4. Interaction of Chick Hp with Human Hb.

In view of the controversy in the literature regarding the presence of Hp in chick plasma exploratory studies were undertaken with regard to the interaction of chick Hp with human Hb.

When chick Hp in 8 week old hen serum was monitored using human Hb instead of chick Hb there was no evidence for the presence of Hp as measured by column chromatography or cellulose acetate electrophoresis (Figure 13, Figure 15b). The inability of the human Hb to combine with the chick Hp was not due to the presence of an inhibitor or denaturation of the Hb as a 50:50 mixture of pig and chick plasma showed binding capacity with human Hb (Figure 15c). This mixture contains half the binding capacity of undiluted pig plasma (Figure 15 d).

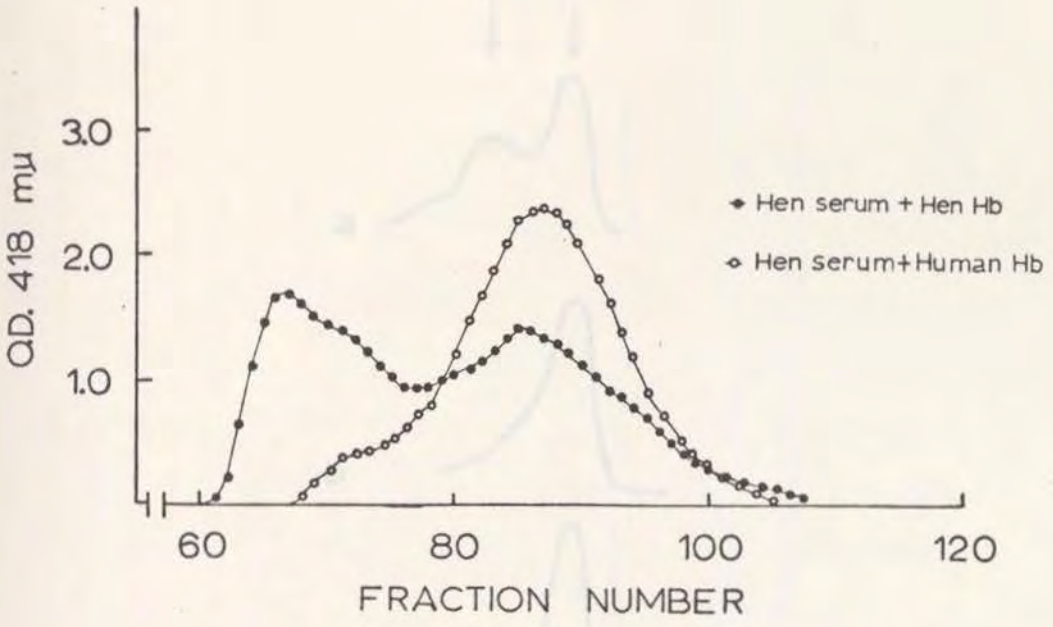


Figure 13: Sephadex G-100 column chromatography of hen serum and hen Hb. An excess of hen Hb was added to hen serum and the sample was chromatographed on a Sephadex G-100 column (2 x 23 cm) equilibrated in 0.9% NaCl. The flow rate was 20 ml/hr.

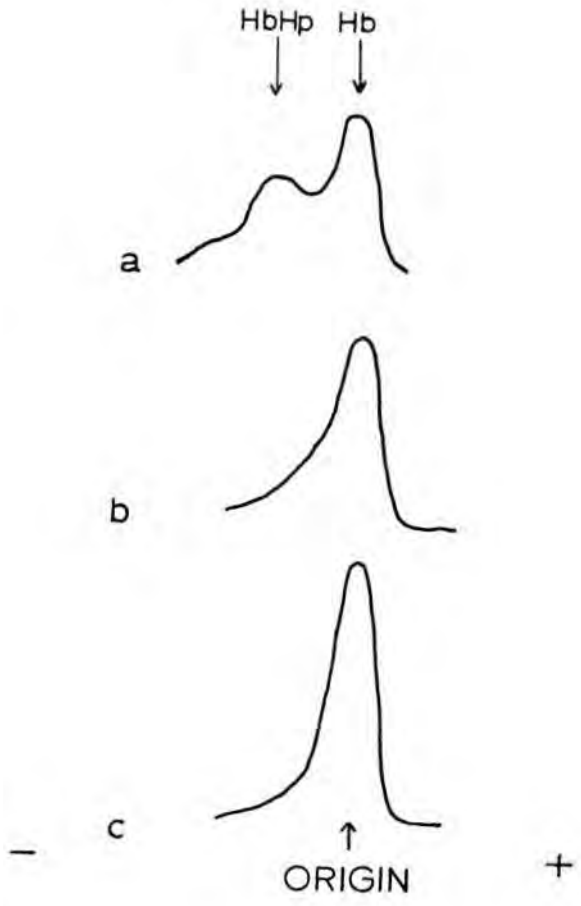


Figure 14: Cellulose acetate electrophoresis profiles of chick plasma samples mixed with hen Hb. Electrophoresis took place at pH 7.0 in .05 M phosphate buffer (2.5 mA/strip) for 30 minutes as described by Pantlitschko and Weippl (1968)

A. 8 week chick plasma plus hen Hb.
B. 21 day chick embryo plasma plus hen Hb.
C. 10 day chick embryo plasma plus hen Hb.

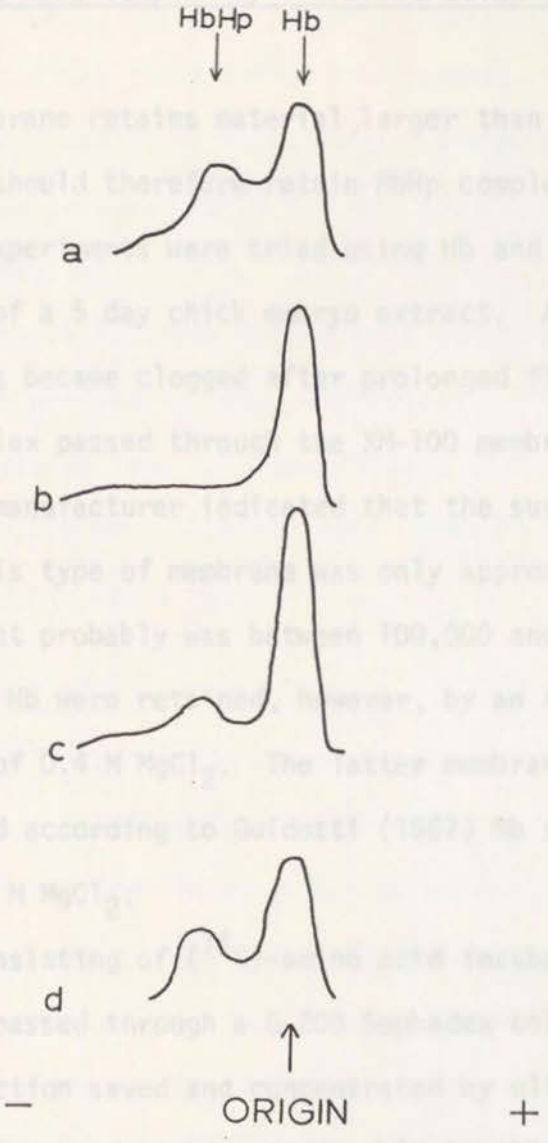


Figure 15: Electrophoresis profiles of chick and pig plasma samples containing either human or hen Hbs. Electrophoresis took place for 30 minutes at pH 7.0 in .05 M phosphate buffer.

A. 8 week chick plasma and hen Hb.
B. 8 week chick plasma and human Hb.
C. 8 week chick plasma and pig plasma (1/1) and human Hb.
D. Pig plasma and hen Hb.

5. Attempt to Isolate HbHp Complex by Ultrafiltration and Column Chromatography.

An XM-100 membrane retains material larger than 100,000 molecular weight and should therefore retain HbHp complexes (MW, 140,000, 175,000). Experiments were tried using Hb and HbHp as well as a 50% supernatant of a 5 day chick embryo extract. Aside from the fact the membranes became clogged after prolonged filtration both Hb and HbHp complex passed through the XM-100 membrane. Discussions with the manufacturer indicated that the suggested cut-off of 100,000 for this type of membrane was only approximate and the actual cutoff point probably was between 100,000 and 250,000 M.W. Both complex and free Hb were retained, however, by an XM-50 membrane even in the presence of 0.4 M $MgCl_2$. The latter membrane has a cutoff point of 50,000 MW and according to Guidotti (1967) Hb should exist as dimer in 0.4 M $MgCl_2$.

Homogenate consisting of (^{14}C)-amino acid incubated embryos (4 somite stage) was passed through a G-200 Sephadex column and the Hb containing fraction saved and concentrated by ultrafiltration. The method proved tedious and we were never able to obtain a HbHp peak distinct and well separated from the large portion of other radio-active proteins.

6. Attempted Precipitation of HbHp Complex by Antiserum

The antiserum used in this study reacted readily with 5 day embryonic Hb-porcine Hp complex. Using double diffusion techniques

the antiserum showed precipitin lines for free Hp and half saturated and fully saturated complexes, but gave no precipitate with either free Hb (Figure 16) or a 5 day chick embryo homogenate. The equivalence point occurred at a concentration of Hp between 0.5 and 0.25 mg/ml. (Figure 17) and the serum produced about 4.0 mg. of antigen antibody precipitate/ml in this region.

The precipitin curve for free Hp and half saturated and fully saturated chick Hb porcine Hp complexes (Figure 18) shows that saturated complex yields less of a precipitate than free Hp. Essentially the same results were obtained when human Hb was used in place of the 5 day embryonic chick Hb.

7. Preparation of Isotopically Labelled Hbs.

In view of the difficulties in obtaining substantial quantities of radiolabelled chick embryo Hb of high specific activity subsequent experiments to further develop the desired assays were made with preparations of either human or 5 day embryonic chick Hb labelled in vitro. For this purpose the Hbs were treated with (^{14}C)-iodoacetamide according to the method of Malchy and Dixon (1969), freed from excess (^{14}C)-iodoacetamide by chromatography on G-25 Sephadex and concentrated. The human Hb had a specific activity of 2.0×10^5 cpm/mg. whereas the chick Hb had a specific activity of 4.0×10^5 cpm/mg.

Both labelled Hbs complexed fully with porcine Hp (Figure 19). A preparation of radioactive Hb from 7 day chick embryos was obtained.

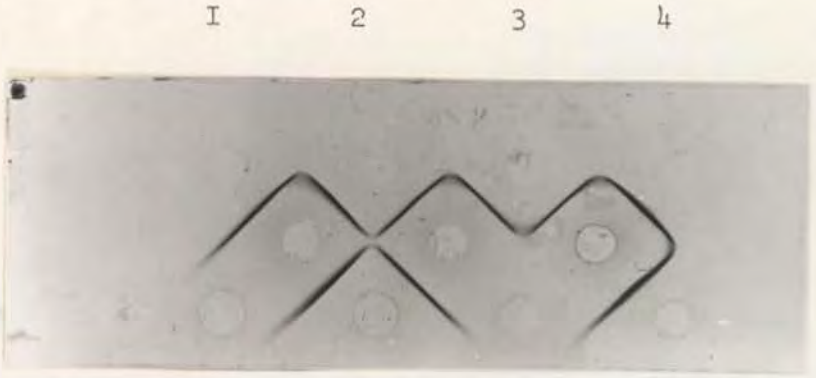


Figure 16: Immunodiffusion of Hp antisera with Hp, HpHb and Hb samples. Ouchterlony plates (0.85% agar) were run in borate saline for 3 days at room temperature in a moist chamber. The samples were stained with 1% Napthal Blue Black. The center wells contained antisera/2 and the numbered wells contained sample as indicated.

	Hp (ɣ/ml)	Hb (ɣ/ml)		Hp (ɣ/ml)	Hb (ɣ/ml)
1.	500	0	5.	0	500
2.	500	60	6.	500	300
3.	250	0	7.	0	250
4.	250	30	8.	250	150

	T	2T	4T	8T	16T
Hp mg/ml	1	0.5	0.25	0.125	0.0625

Anti-Hp/2 ⟶

Figure 17: Precipitin titration of anti-Hp/2. Ouchterlony plates were run in borate saline for 3 days at room temperature in a moist chamber. Samples were stained with 1% Napthal Blue Black. The equivalence point occurs at a concentration of 0.25 mg/ml Hp. At this point the precipitin line is the sharpest and also equidistant between the antigen and antibody wells.

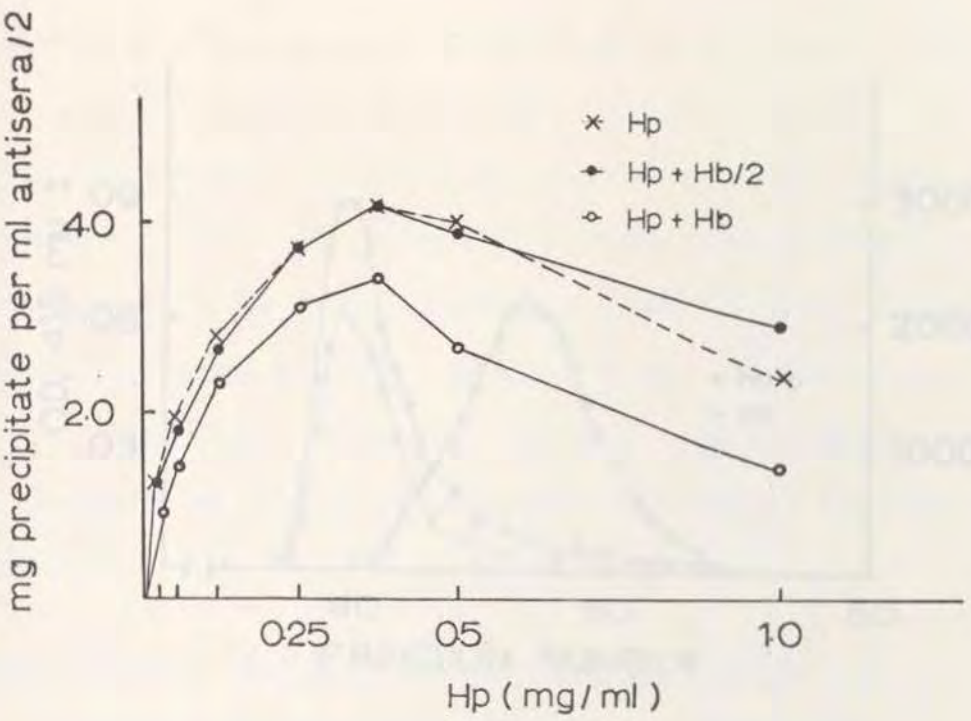


Figure 18: Precipitin curves of pig Hp alone or mixed with embryonic chick Hb plus antisera. Half saturating and saturating amounts of 5 day embryonic chick Hb were added to pig Hp. After addition of antisera/2 and incubation for a half hour at 37°C samples were stored at 0° C for 72 hours; precipitates being resuspended each day. After centrifugation and washing amount of protein in the precipitates was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

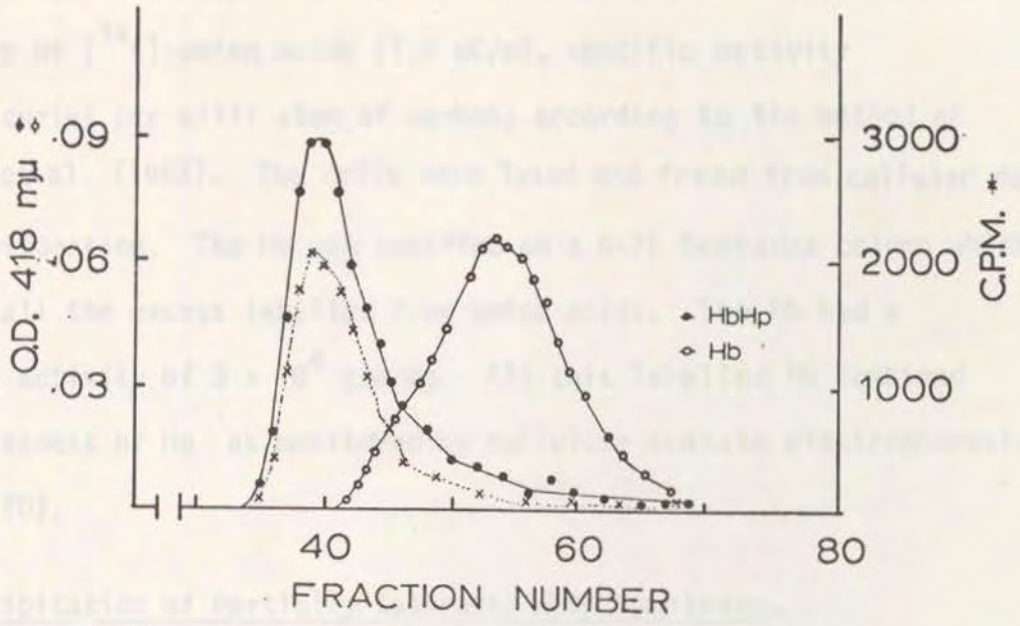


Figure 19: Sephadex G-100 column chromatography of a mixture of Hp and an unsaturating amount of [^{14}C] labelled chick Hb. Samples were chromatographed on a Sephadex G-100 column equilibrated in borate saline (flow rate was 20 ml/hr). The profile of Hb alone is added as a reference.

by incubation of a suspension of erythroid cells for 4 hours with a mixture of (^{14}C)-amino acids (1.0 uC/ml, specific activity 40 millicuries per milli atom of carbon) according to the method of Borsook et al. (1957). The cells were lysed and freed from cellular debris by centrifugation. The Hb was purified on a G-75 Sephadex column which removed all the excess labelled free amino acids. The Hb had a specific activity of 3 x 10^4 cpm/mg. All this labelled Hb combined with an excess of Hp as monitored by cellulose acetate electrophoresis (Figure 20).

8. Precipitation of Partially Saturated HbHp Complexes.

As it was proposed to precipitate Hb from blastodiscs using an excess of Hp all further experiments were performed with unsaturated complexes formed by treatment of the purified Hbs with an excess of Hp.

Between 70-90% of unsaturated human Hb complexes were precipitated by the Hp antiserum when the amount of HbHp remaining in solution was measured either by difference spectra or by radiochemical techniques. (Tables 1 and 2).

However when chick Hb was used instead of human Hb only between 30 and 50 per cent of the HbHp was removed from solution at the equivalence point after addition of antisera/2. These results were similar for unlabelled and both types of labelled Hbs. (Tables 3, 4, 5).

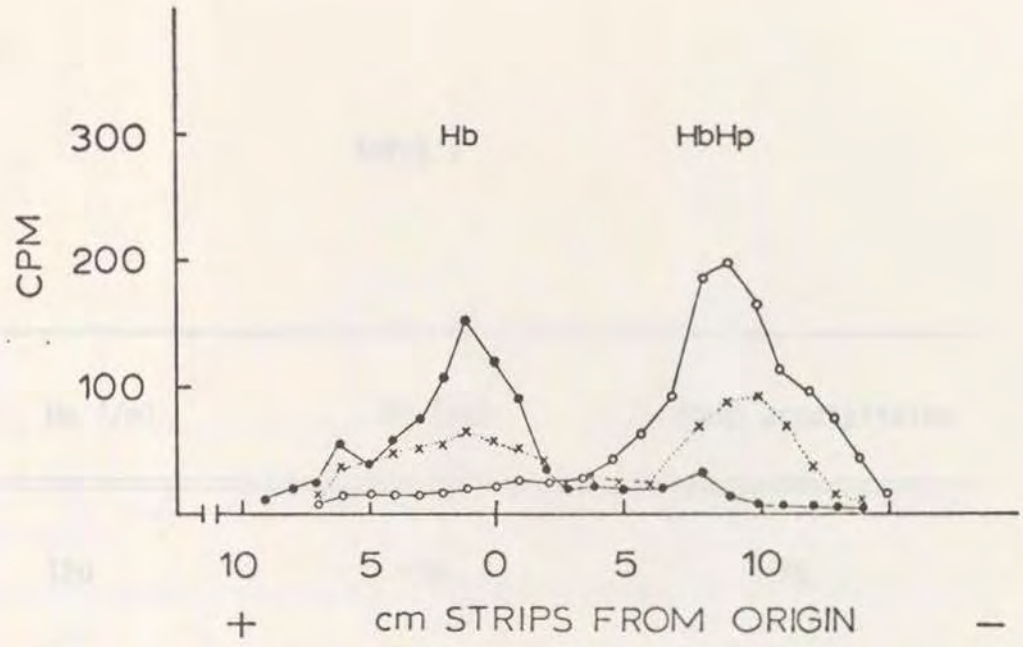


Figure 20: Cellulose acetate electrophoresis of /^{T4}C/ chick Hb and Hp samples. The 7 day embryo chick Hb was prepared by the in vitro method of Borsook et al. (1957) After electrophoresis (see Figure 14 for conditions) bands with a width of 1 cm were cut from the cellulose acetate strips and counted in Toluene-PPO-POPOP scintillation cocktail. This is a composite graph of 3 separate electrophoretic runs containing:

- • Hb alone
- × Hb + HbHp
- ◦ Hp + HbHp

TABLE 1

Hp ɣ/ml	Hb ɣ/ml	% HbHp precipitated
120	50	79
100	50	63
80	50	64

Table 1:

Precipitation of human Hb porcine Hp complex with anti-Hp. Hp antisera/2 was added to equal volumes of sample containing human Hb and an excess of pig Hp in borate saline (pH 8.4) and incubated for 30 minutes at 37° C. After centrifugation the amount of Hb containing material left in the supernatant was measured by the method of Levere and Granick (1967).

TABLE 2

	Hp γ/ml	Human Hb γ/ml	% of HbHp precipitated
2T	500	250	77
4T	250	125	84
8T	125	62.5	73
16T	62.5	31.25	26

Precipitation of /^{T4}C/ human Hb porcine Hp with anti Hp. The radioactivity remaining in the supernatant after addition of antisera/2 indicated the percent Hb containing material precipitated.

TABLE 3

Hp ɣ/ml	Hb ɣ/ml	% HbHp Precipitated
250	25	44
250	63	52
250	80	45
250	149	52

Precipitation of chick Hb porcine Hp complexes with anti Hp as measured by difference spectra techniques. 5 day embryonic chick Hb was added to an excess of Hp prior to addition of antisera/2. Hb left in solution after centrifugation was measured by the difference spectra techniques of Levere and Granick (1967).

TABLE 4

	Hp ɣ/ml	500	250	125	62.5
Hb ɣ/ml	Total cts.		% HbHp precipitated		
50	6208	23	32	20	24
25	3042	20	31	26	23
12.5	1492	20	36	25	26
6.25	756	34	44	31	31

Precipitation of /†4C/ chick Hb pig Hp complexes with Hp antisera/2 when Hb was labelled with /†4C/ iodoacetamide. An excess of Hp was added to /†4C/ chick Hb (5 day embryo) prior to the addition of Hp antisera/2. After centrifugation the amount of radioactivity remaining in the supernatant was used to calculate the percent of initial Hb precipitated.

TABLE 5

Hp ϒ/ml	Hb ϒ/ml	Supernatant cpm	Supernatant After Precipitation	% HbHp Pre-cipitated
250	100	743	483	35
250	50	312	204	35
250	25	160	106	33
250	10	103	45	56
250	5	42	21	50

Precipitation of chick /T4C/ Hb porcine Hp complex when the Hb was labelled by the in vitro technique of Borsook et al. (1957). The radioactivity remaining in the supernatant after addition of Hp antisera/2 was used to calculate the % Hb precipitated.

Different precipitating conditions were tried but the results remained essentially the same. With decrease in pH there was a 9% increase in the amount of label precipitated (Table 6). An increase of the Hp concentration to 375 γ/ml from 250 γ/ml resulted in a slight increase in the radioactivity precipitated but increasing the saline concentration had no effect (Table 7). With the Hp concentration remaining constant (250 γ/ml) and Hb at 130 γ/ml and 65 γ/ml , the amount of label precipitated remained the same in each case (Table 8).

The label remaining in solution was tested to see whether or not a further portion of it could be precipitated after addition of first Hp and then antisera/2 (Table 9). Even though the same amount of material was precipitated in each case no counts were removed on the second precipitation steps.

9. The Stability of the Chick Hb Pig Hp Complex

As a difference was shown between the human HbHp complex and chick HbHp with respect to the amounts of Hb containing material that could be precipitated by Hp antisera/2 the stability of both complexes was investigated.

An unsaturating amount of labelled human Hb was added to pig Hp. After a half hour an excess of non-radioactive human Hb was added and the HbHp complex was separated from free Hb by column chromatography. No label appeared in the free Hb peak indicating the stability of the human Hb pig Hp complex (Figure 21).

TABLE 6

pH	Hp + chick Hb % Precipitated	Hp + human Hb % Precipitated
8.4	48	83
7.4	49.5	85
7.0	55	87
6.5	57	87

Effect of varying the pH on the precipitation of HbHp complexes by Hp antisera/2. Hp was at a concentration of 250 γ/ml and Hb at 65 γ/ml. The Hbs were labelled with /^{T4}C/ iodoacetamide.

TABLE 7

Saline Conc. (gms %)	Chick Hb/Hp:I/3 % Precipitated	Chick Hb/Hp:I/2 % Precipitated
0.9	56.2	54.9
1.9	55.2	53.4
3.8	57.6	55.8
7.5	55.9	54.4

Effect of varying the concentration of saline on the precipitation of chick HbHp complexes by Hp antisera/2. The Hp was at a concentration of 375 γ/ml and the 5 day embryonic chick Hb at 130 γ/ml and 170 γ/ml. The Hb was labelled with /^{T4}C/ iodoacetamide.

TABLE 8

Hp γ/ml	Hb γ/ml	% Sat.	Hp	Hpαβ	$Hp(\alpha\beta)_2$	% Precipitated
250	130	74	.07	.39	.54	44
250	65	37	.40	.46	.14	45

Effect of varying the ratios of chick Hb to porcine Hp on the precipitation of /^{T4}C/ label by Hp antisera/2. The Hp was at a concentration of 250 γ/ml and the Hb at 130 γ/ml and 65 γ/ml respectively. The amounts of Hp, Hpαβ and $Hp(\alpha\beta)_2$ present with each concentration of Hb were calculated using the equation $p^2 + 2pq + q^2$ where p = q-1 and q = the % Hp saturated with Hb. p^2 = Hp, 2 pq = Hpαβ and q^2 = Hp $(\alpha\beta)_2$.

Table 9:

The precipitation of chick HbHp complexes by two successive additions of Hp antisera/2. The amount of protein precipitated was measured by the method of Lowry et al. (195 I) using bovine serum albumin as standard. The Hb was labelled with /^{T4}C/ iodoacetamide. The protocol for the experiment was as follows:

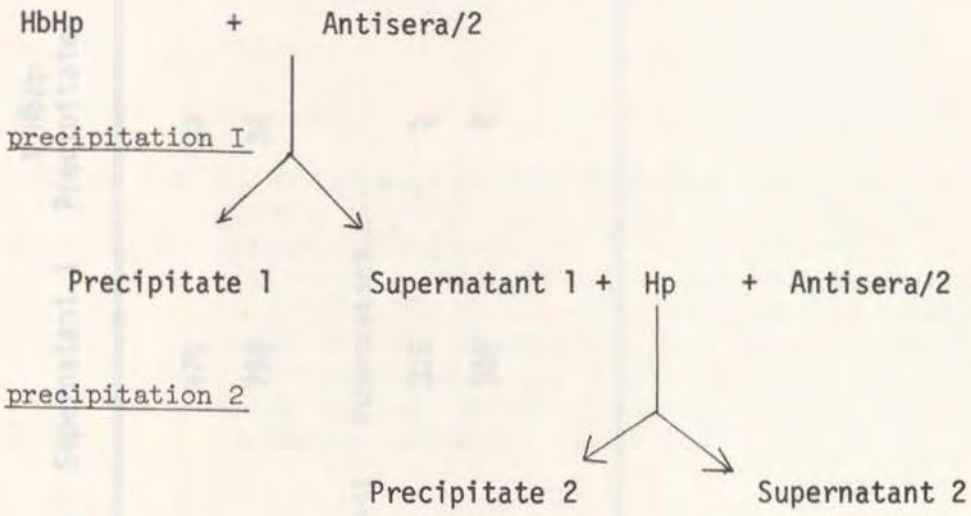


TABLE 9

Hb (γ/ml)	Hp(γ/ml)	Control	Supernatant 1	% HbHp Precipitated	cpm in Precipitate	mg protein precipitated /ml antisera/2
50	250	692	470	32	5600	3.7
100	250	1197	795	34	11,900	3.8
		SupernatantI	Supernatant 2			
35	250	310	305	2	15	3.8
70	250	525	506	4	24	4.0

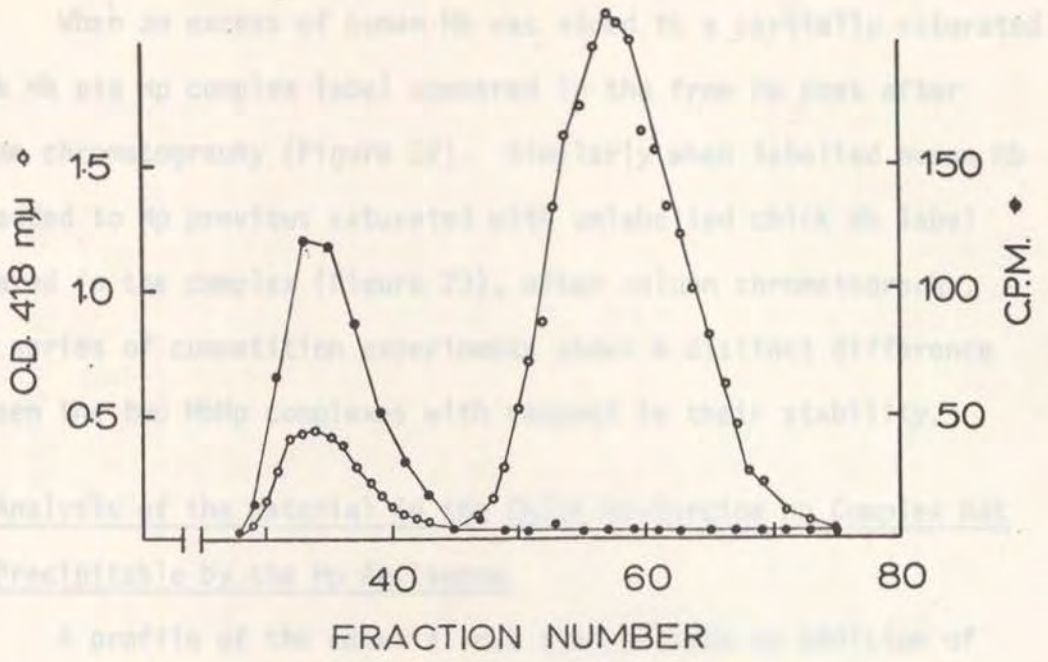


Figure 21: G-100 Sephadex column chromatography of a mixture of /^{T4}C/ human Hb porcine Hp complex and unlabelled human Hb. To a solution containing porcine Hp and an unsaturating amount of /^{T4}C/ human Hb was added an excess of unlabelled human Hb. The mixture was left overnight and chromatographed at a flow rate of 20 ml/hr on a G-100 Sephadex column (1.5 x 30 cm) equilibrated in borate saline.

When an excess of human Hb was added to a partially saturated chick Hb pig Hp complex label appeared in the free Hb peak after column chromatography (Figure 22). Similarly when labelled human Hb was added to Hp previous saturated with unlabelled chick Hb label appeared in the complex (Figure 23), after column chromatography. This series of competition experiments shows a distinct difference between the two HbHp complexes with respect to their stability.

10. Analysis of the Material in the Chick Hb-Porcine Hp Complex not Precipitable by the Hp Antiserum.

A profile of the material not precipitable on addition of antiserum to chick Hb-porcine Hp complex was determined by G-200 Sephadex column chromatography (Fig. 24). A portion of the radioactive label appeared in a peak corresponding to soluble antigen-antibody complex. However most of the label was recovered as apparent HbHp.

It seemed possible that the chick Hb might be displaced from the pig Hp and bound to the rabbit Hp present in the antiserum. The rabbit antisera contained 54 mg Hp%. Thus the antisera/2 would contain 270 γ Hp/ml, a concentration slightly above the porcine Hp of 250 γ/ml. Therefore, an attempt was made to suppress any such transfer by a preliminary treatment of the antiserum with an excess of non radioactive labelled human Hb. However when the material not precipitable by addition of this treated antiserum was examined by Sephadex chromatography radioactivity was recovered in the peak

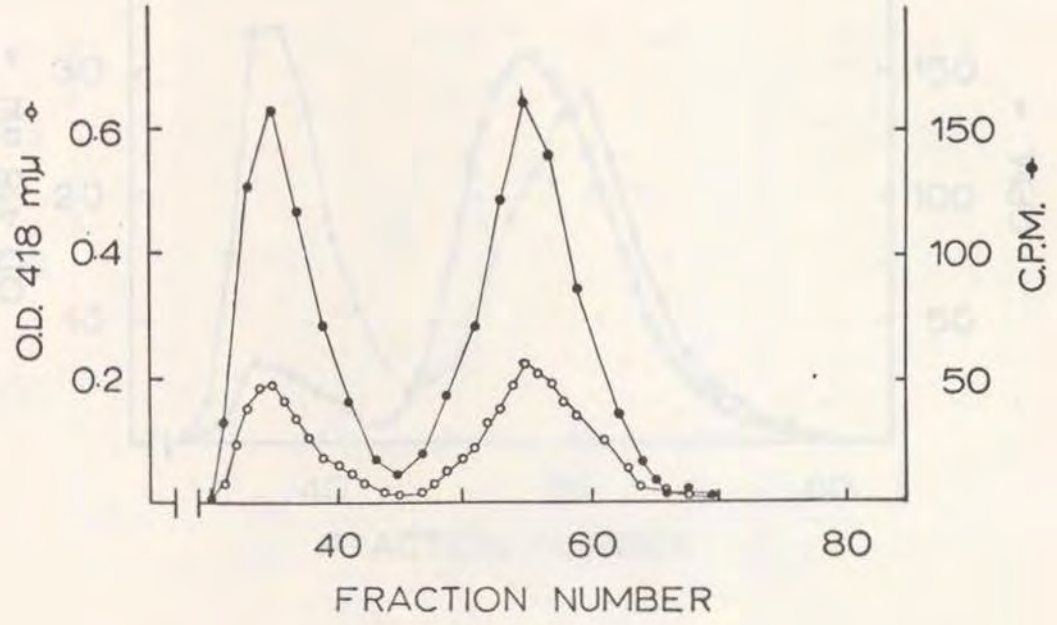


Figure 22: G-100 Sephadex column chromatography of a mixture of /^{T4}C/ chick Hb porcine Hp complex and unlabelled human Hb. To a solution containing porcine Hp and an unsaturating amount of 5 day embryonic chick /^{T4}C/ Hb was added an excess of unlabelled Hb. The mixture was left overnight and chromatographed at a flow rate of 20 ml/hr on a G-100 Sephadex column (1.5 x 30 cm) equilibrated in borate saline.

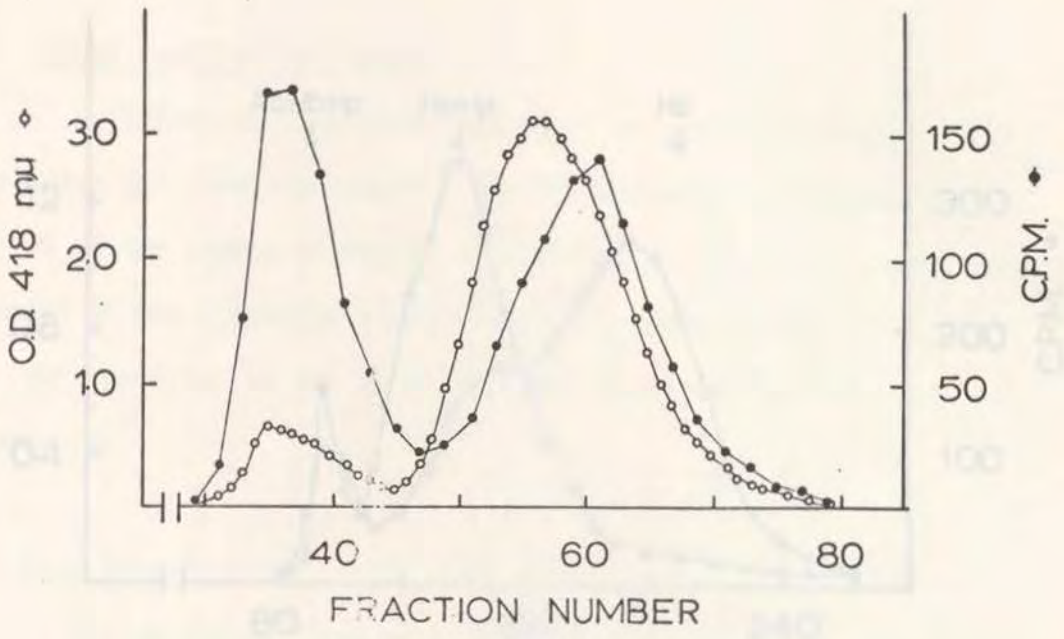


Figure 23: G-100 Sephadex column chromatography of a mixture of chick Hb porcine Hp complex and /^{125}I/ human Hb. To a solution containing porcine Hp and an excess of unlabelled 5 day chick Hb was added /^{125}I/ labelled human Hb. The mixture was left overnight and chromatographed on a Sephadex G-100 column equilibrated in borate saline.

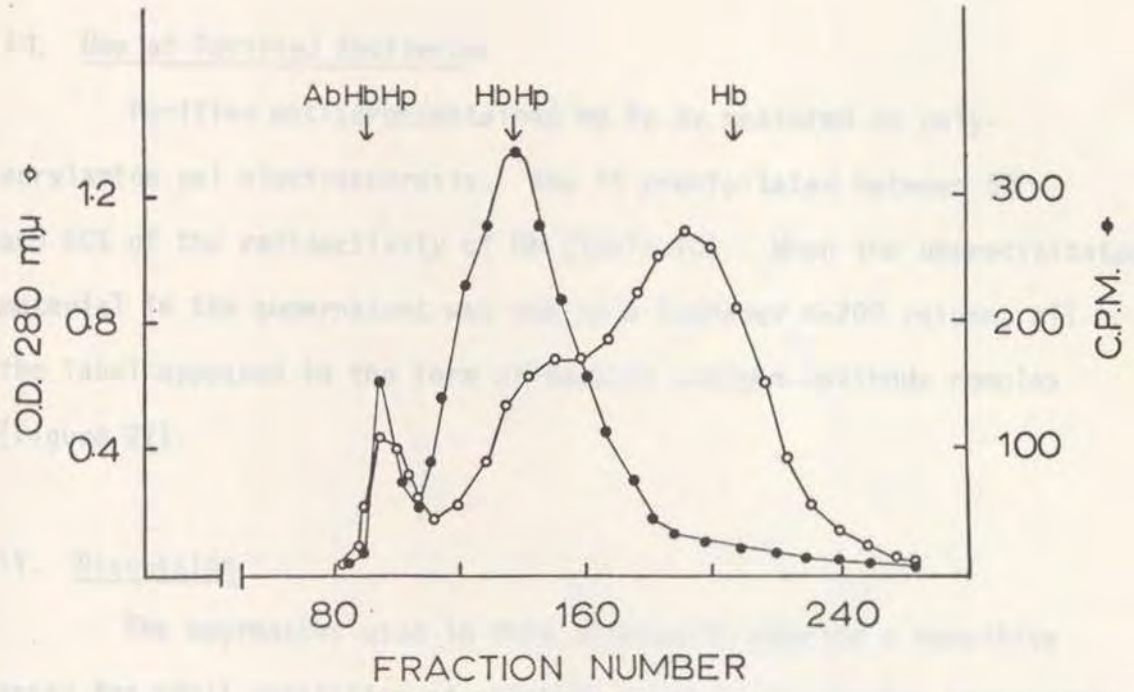


Figure 24: G-200 Sephadex column chromatography of the supernatant fraction from a mixture containing /T4C/ labelled 5 day embryonic chick Hb, an excess of pig Hp and Hp antisera. Rabbit antisera against Hp was added to a mixture containing pig Hp (250 ɣ/ml) and /T4C/ labelled chick Hb (130 ɣ/ml). After incubation at 37° C for 30 minutes the sample was centrifuged and 44% of the labelled Hb was precipitated. The supernatant was chromatographed on a Sephadex G-200 column (2.3 x 33 cm) equilibrated in borate saline. The flow rate was 40 ml/hr.

of free Hb (Figures 25, 26).

11. Use of Purified Antiserum

Purified antiserumcontained no Hp as measured on polyacrylamide gel electrophoresis. Now it precipitated between 50 and 60% of the radioactivity of Hb (Table 10). When the unprecipitated material in the supernatant was run on a Sephadex G-200 column, all the label appeared in the form of soluble antigen-antibody complex (Figure 27).

IV. Discussion

The approaches used in this attempt to develop a sensitive assay for small quantities of chick Hb in crude blastodisc homogenates were based on two assumptions. First it was supposed that the blastodisc does not itself contain significant quantities of Hp. The absence of detectable quantities of Hp in the circulation of 10 day embryos (Figure 14c) is strong evidence that this assumption is valid. However,a more rigorous demonstration would have been desired had the assay procedure been successfully developed.

Parenthetically, the preliminary observation on the Hp found in the circulation of 8 week old chicks (Figure 14a) may shed some light on the controversy in the literature regarding the question of whether Hps are present in the circulation of the chick. My demonstration of the presence of Hp in chick serum is in accord with Darcel's (1969) demonstration of Hp in 10 week old chicks by

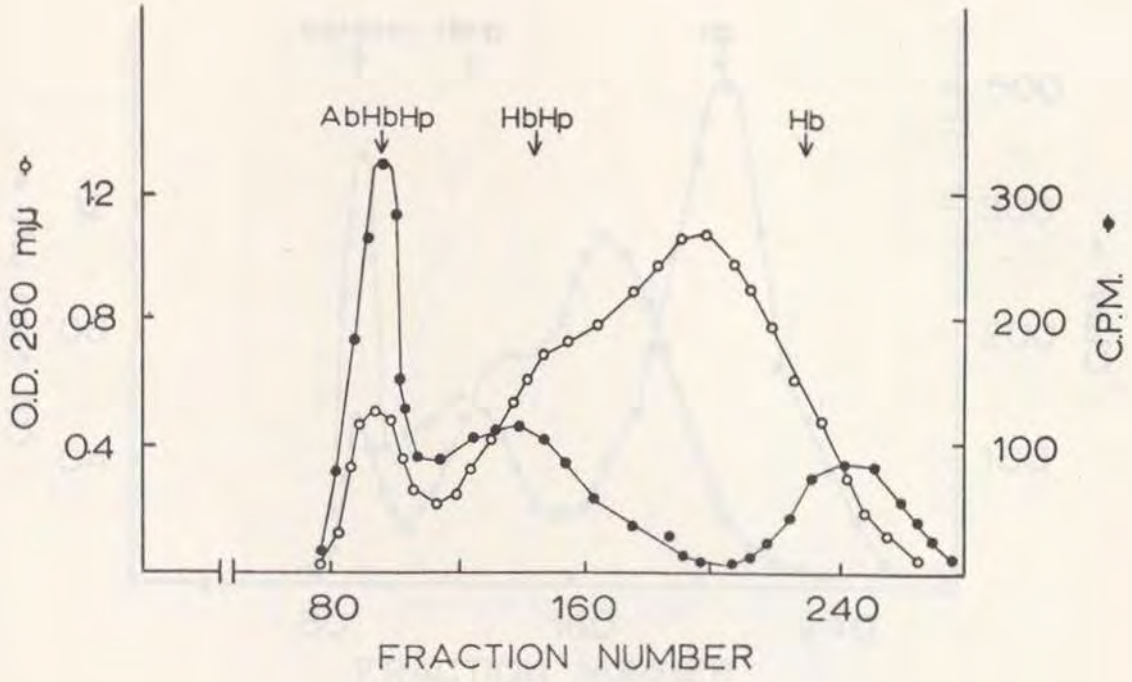


Figure 25: G-200 Sephadex column chromatography of the supernatant fraction from a mixture containing ^{125}I^{14}C labelled 5 day embryonic chick Hb, an excess of pig Hp, human Hb, and Hp antisera. Rabbit antisera against Hp was added to a mixture containing pig Hp (250 ɣ/ml), labelled chick Hb 65 ɣ/ml, human Hb 175 ɣ/ml and incubated at 37^{o} C for 30 minutes. After centrifugation 50% of the radioactivity was precipitated. The supernatant was added to a G-200 Sephadex column (2.3 x 33 cm) equilibrated in borate saline and chromatographed at a flow rate of 40 ml/hr.

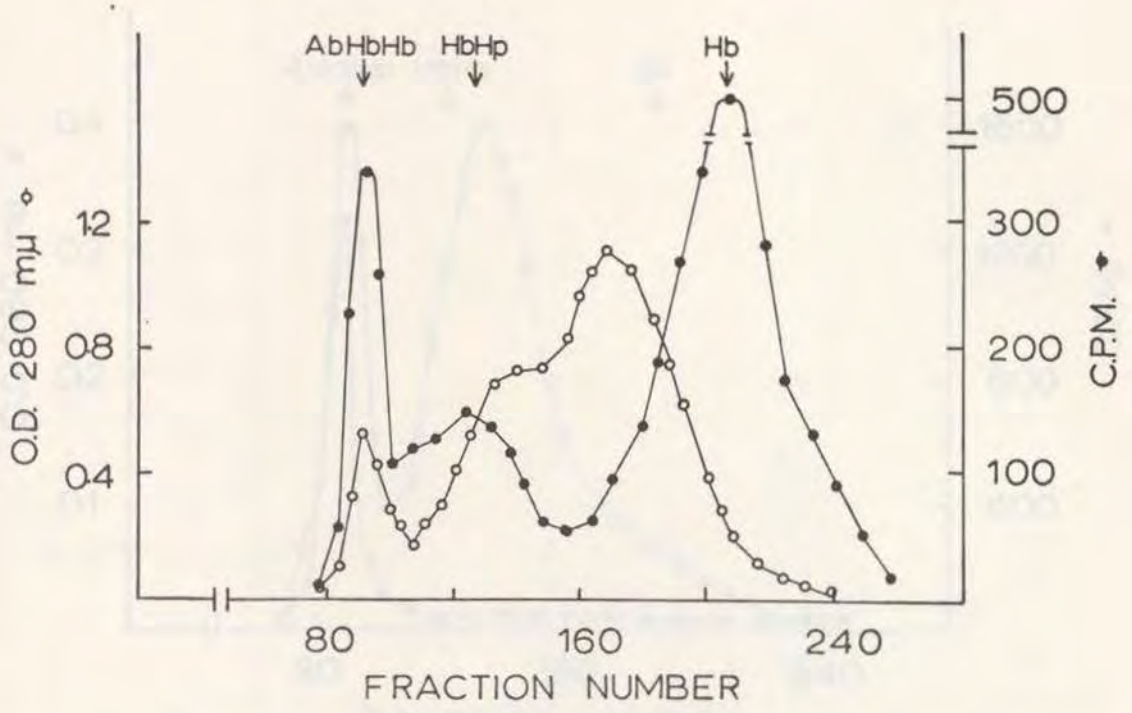


Figure 26: G-200 Sephadex column chromatography of the supernatant fraction from a mixture containing /T4C/ labelled 5 day embryonic chick Hb, an excess of both pig Hp and human Hb, and Hp antisera 35% of the radioactivity was precipitated from a solution containing Hp (250 ɣ/ml), /T4C/ labelled chick Hb 65 ɣ/ml, human Hb (350 ɣ/ml) after addition of Hp antisera. The supernatant was chromatographed on a Sephadex G-200 column (2.3 x 33 cm) equilibrated in borate saline with a flow rate of 40 ml/hr.

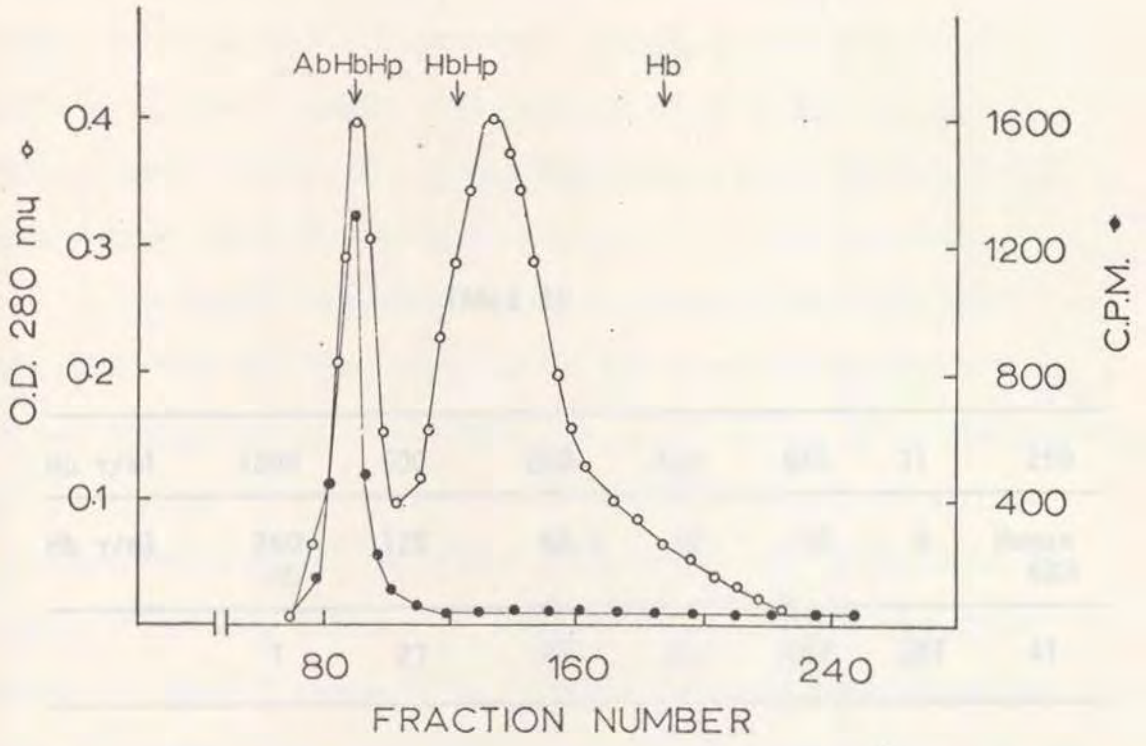


Figure 27: G-200 Sephadex column chromatography of the supernatant fraction from a mixture containing [^{14}C] labelled 5 day embryonic chick Hb, an excess of pig Hp and purified Hp antisera. 41% of the radioactivity was precipitated from a solution containing Hp (250 ɣ/ml) and Hb (31 ɣ/ml) after the addition of purified Hp antisera. The supernatant was chromatographed on a Sephadex G-200 column (2.3 x 30 cm) equilibrated in borate saline with a flow rate of 40 ml/hr.

TABLE 10

Hp γ/ml	1000	500	250	125	625	31	250
Hb γ/ml	250γ Hb	125	62.5	31	16	8	Human 62.5
	T	2T	4T	8T	16T	32T	4T
Control	9042	4318	2201	1083	544	268	1173
After Precipitation	7457	2406	831	593	323	194	54
% Precipitated	17	45	62	45	41	38	95.5

Precipitation of Hp complex with 5 day embryonic chick Hb using purified antisera/2. The antisera was purified as described in methods. The Hb was labelled with /T4C/ iodoacetamide.

formation of complex with chick Hb. Conversely the failure of chick Hp to form a complex with human Hb (Figure 15b) may explain the conclusion of Riou et al. (1962) that chick plasma contains no Hp on the basis of failure to observe complex formation with horse Hb.

The second foundation for my experimental approach was the assumption that the parameters of the reactions between Hb and Hp and the properties of the resulting complexes are mainly independent of the sources of the reactants. The validity of this assumption is generally accepted. Indeed as recently as 1973 in reporting the complexing of only 50% of a preparation of adult chick Hb with an excess of human Hp Cohen-Dix et al. (1973) summarily dismissed the lack of a quantitative reaction as indicative of denaturation of the Hb preparation.

However, the preliminary data obtained in this study clearly establish that the assumption is incorrect. As noted above, chick Hp failed to form a complex with human Hb. Conversely, chick Hb did not form stable complexes with porcine Hp., (Figures 25,26). In addition, chick Hb could be displaced from pre-existing complex by addition of human Hb (Figures 22, 23) whereas chick Hb cannot displace human Hb from pig Hp complex. Furthermore, in contrast to the complex formed between mammalian Hb and Hp a substantial portion of the complex containing chick Hb formed a soluble complex with anti-Hp antibodies (Tables 3,4,5, 10, Figure 24).

These differences in properties between chick Hb-mammalian Hp complexes and those of the typical mammalian Hb-mammalian Hp complex

have precluded successful development of the desired assay method by the particular approaches used. Nevertheless, in the light of the unexpected properties of the chick Hb-porcine Hp complexes which I have demonstrated it still seems possible that an assay based on the precipitation of HbHp complexes can be devised using homologous complexants and a more specific antiserum. The approach adopted has in fact already yielded a satisfactory method of microassay of globin chains of mammalian Hbs synthesized de novo for the model system in which all components were from mammalian sources.

Confirmation of the presence of Hp in chick plasma (Figure 14) and the demonstration it will form complexes with chick Hb (Figure 13) now reopen the possibility of isolating the small quantity of Hb in the blastodisc as the chick Hp complex. Further there is no a priori reason to suppose that antibodies formed specifically against adult chick Hp would yield a soluble antigen-antibody complex with chick Hb chick Hp.

However, examination of these possibilities will first require development of methods for isolation of homogeneous preparations of chick Hp. Therefore in view of the uncertainties involved in this proposal the project was temporarily discontinued at this stage.

V. Summary and Conclusions

1. Both adult and fetal chick Hbs can bind to porcine Hp as measured by column chromatographic and electrophoretic techniques.

2. A rabbit antiserum against pig Hp can precipitate up to 90% of human Hb in the presence of excess Hp.

3. This same antiserum is unable to precipitate more than 60% of embryonic chick Hb from solution even in the presence of excess Hp.

4. Reasons for this incomplete precipitation appear to be two-fold. First, although chick Hb will bind to pig Hp the binding is weak and therefore some of it may bind to rabbit Hp present in the antisera. Secondly, even when no rabbit Hp is present a soluble HpHb antibody complex is formed.

5. Competition studies show that the human Hb pig Hp complex is quite stable whereas the chick HbHp complex is much weaker as human Hb can displace it from the Hp molecule.

6. The chick does not appear to have a Hp until after hatching.

7. Preliminary experiments suggest that human Hb is unable to bind successfully with hen Hp, even though it forms complex with hen Hb.

Project II

Attempted development of an assay for the determination of the mitochondrial enzyme δ aminolevulinic acid synthetase in the 6-8 somite chick blastodisc.

I. INTRODUCTION

A rapid rise in Hb synthesis occurs between the 6-7 somite stages of chick embryonic development. This rapid rise appears to be regulated by several factors including the supply of δ amino-levulinic acid (ALA). Supplements of ALA added to chick blastodiscs lead to a stimulation of Hb production, (Levere and Granick, 1965; Wilt, 1965, 1967; Wainwright and Wainwright 1966, 1970). Synthesis of this key intermediate from succinyl CoA and glycine by mitochondrial ALA synthetase is the rate limiting step in the formation of heme prosthetic groups. As a result a study of the ontogeny of this enzyme and the factors controlling its activity would be an important addition to the understanding of one of the mechanisms involved in the control of Hb synthesis. This investigation would initially require a sensitive assay for ALA synthetase.

Mauzerall and Granick (1956) have reported a colorimetric assay for this enzyme. It was not sensitive enough for an accurate determination of enzyme activity in the young chick blastodisc but did appear to indicate the presence of barely detectable amounts of ALA synthetase. Both Strand et al. (1972) and Freshney and Paul (1970) have described radiochemical assays of much greater sensitivity which were therefore expected to be sensitive enough to monitor ALA synthetase in the chick blastodisc developing between the primitive streak and 8 somite stages.

These assays were first tried on tissues producing Hb (6-8 somite blastodiscs) which were expected to contain maximal ALA synthetase activity. Unfortunately activities of the enzyme were so low that the measurement of ALA synthetase at earlier stages was impossible and as a result the project was abandoned.

II. METHODS

Two methods for measuring ALA synthetase were examined. In one /2 - ^{14}C/ glycine (20.8 mC/mM, N.E.N.) was used as substrate at a concentration of 12 μC/ml of incubation mixture as described by Freshney and Paul (1970). Twenty-four embryo (6-8 somite stage) were homogenized in 0.8 ml of 0.05 M phosphate buffer. 500 λ of this homogenate was added to an equal volume of double strength incubation mixture and incubated for 4 hours at 37^{o} C. The reaction was stopped by the addition of 0.100 ml 15 mM ALA, 0.25 ml 25% TCA and 0.10 ml 15 mM glycine. After centrifugation 100 λ of the deproteinized supernatant was spotted onto a 15 cm x 20 cm silica gel thin layer sheet (Malinkrodt "Chromar 1000" glass fiber mat, impregnated with silica gel; total thickness 1mm; Canlab, Cambridge) and subjected to electrophoresis at 400V and 80 mA for three hours. Electrophoresis was in 0.05 M pthalate buffer maintained from 3^{o} C to 5^{o} C. The sheet was dried and stained with ninhydrin. ALA spots were eluted and counted, or strips from the sheet were cut

out and counted. Radioactivity was measured in vials containing Toluene-PPO-POPOP scintillation cocktail by standard techniques.

The incubation mixture of Strand et al. (1972) containing 5 μC/ml [2,3-^{14}C] succinate [9.81 mC/mM N.E.N.] was also used. A volume of 0.5 ml of an homogenate of 6-8 somite embryos was added to double strength incubation mixture. After incubation for 1 hour at 37° C the reaction was stopped by the addition of a 0.2 ml solution containing 25% TCA, 12.5 μM Na succinate and 125 mu moles ALA. The pH of the incubation mixture was adjusted to 7.0 with NaOH. It was then passed through a Dowex 1 acetate column (1 x 4 cm) equilibrated to pH 7.0 with .025 M Na acetate which was placed in tandem with a column of Dowex 50 H^+ (1 x 4 cm) equilibrated to pH 2.0 with .01 N HCl. After a wash of 20 ml water the Dowex 1 column was discarded. The Dowex 50 H^+ column was washed with 20 ml 0.1 N HCl followed by 6 ml 1 M Na acetate. The ALA fraction was eluted with 8 ml of 1 M Na acetate and subsequently converted to the pyrrole (2 methyl-3-acetyl-4-(3-propionic acid)) by boiling 20 minutes with 0.2 ml acetylacetone at pH 4.5. This pyrrole was applied to a Dowex 1 acetate column (1 x 1.5 cm) equilibrated to pH 4.6 with .05 M Na acetate, washed with 15 ml H_2O, 5 ml of 1 N acetic acid, 1 ml methanol and eluted with 4 ml methanol/glacial acetic acid (2:1). The ALA pyrrole was dried under vacuum dissolved in methanol added to a counting vial and the radioactivity was counted as previously described in a Toluene-PPO-POPOP scintillation cocktail.

III. RESULTS

After three hours of high voltage electrophoresis (Freshney and Paul, 1970) [^{14}C] ALA standard was separated from unlabelled glycine (Figure 28). Moreover, a sample of [^{14}C] ALA containing as few as 500 cpm could be detected and separated from the amount of [2-^{14}C] glycine used in the reaction mixture (Figure 29). However no radioactivity was present in the ALA spot after the homogenate had been incubated at 37^{o} C for 4 hours in the presence of [2-^{14}C] glycine, even though aliquots of reaction mixture equivalent to 1.5 embryos were subjected to electrophoresis at one time.

Using the method of Strand et al. (1972) homogenates containing aliquots equivalent to 5 to 10 embryos (6-8 somite stage) contained only low activities of ALA synthetase. Counts were only double the background (Table 11), even though succinate of the highest available specific activity was used.

IV. DISCUSSION AND CONCLUSION

Using both staining and colorimetric assay techniques Levere et al. (1967) reported the presence of barely detectable amounts of ALA synthetase in individual young chick blastodiscs. Using more sensitive radiochemical techniques we were only able to detect low ALA activity at a stage of embryological

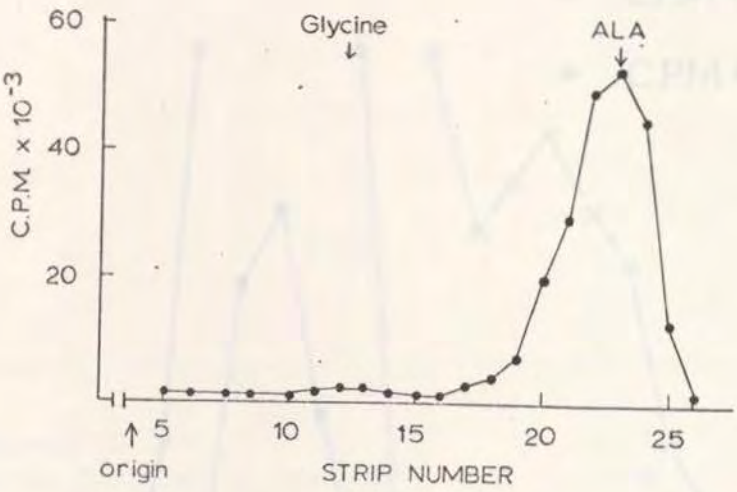


Figure 28: Separation of ^{14}C ALA from unlabelled glycine by high voltage paper electrophoresis for 3 hours at 120-160 mA in .05 M pthalate buffer (pH 4.0). A 10 λ sample was spotted at the origin and glycine and ALA were stained with ninhydrin (positions indicated by arrows in the figure). The chromatogram was cut in 0.25 inch strips which were counted by standard techniques.

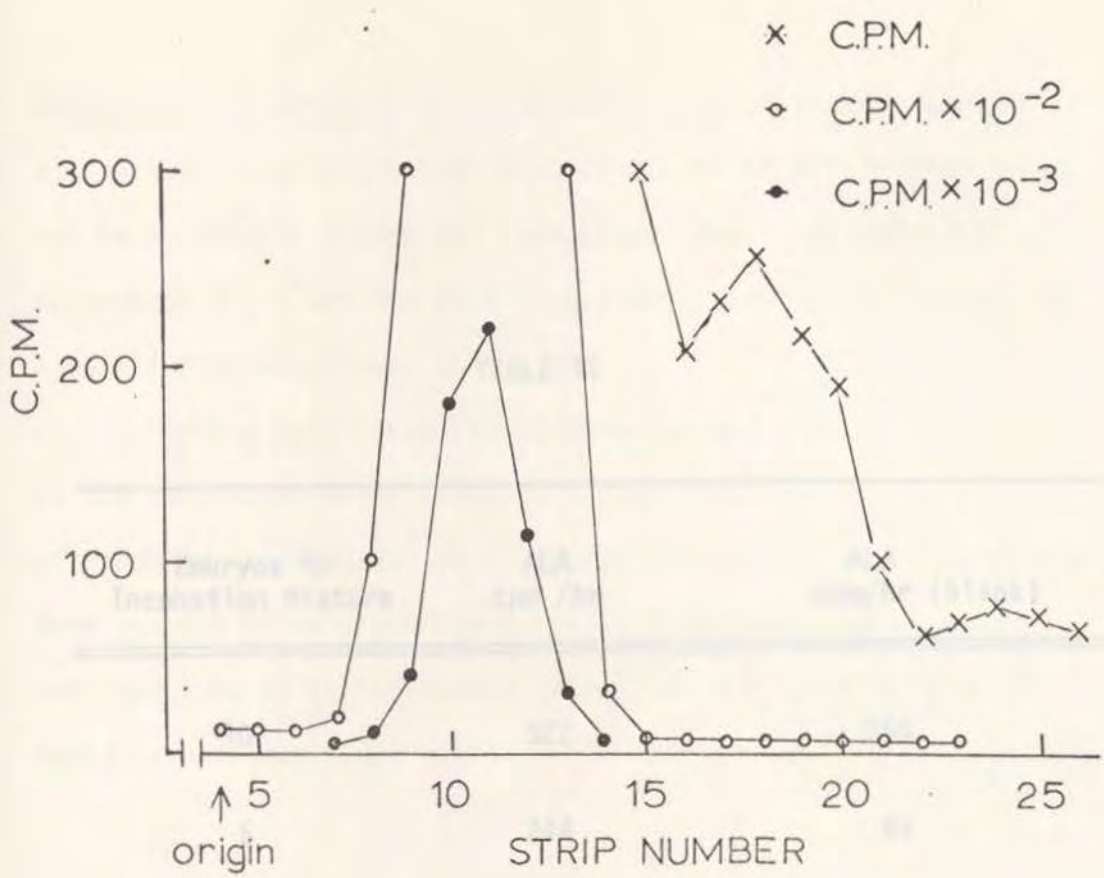


Figure 29: The separation of [^{14}C] ALA (500 CPM/100 λ) from [14C] glycine present in the incubation mixture. Strips (0.25 inches) were cut from electropherogram and counted as described in Methods. The three curves are the same data plotted against different ranges of CPM on the abscissa.

TABLE 11

Embryos in Incubation Mixture	ALA cpm /hr	ALA cpm/hr (blank)
10	322	165
5	244	87
0	157	0

ALA synthetase activity in 6-8 somite embryos, measured as cpm ALA formed per hour. The enzyme was measured essentially by the method of Strand et al. (1972). [2,3-^{14}C]succinate (9.8 mC/mM N.E.N.) was present in the incubation mixture at a concentration of 5 μC/ml. Incubations were for a period of 1 hour at 37° C. Labelled ALA was separated from [2,3-^{14}C]succinate by conversion to the pyrrole and column chromatography as described in methods.

development at which Hb is being rapidly synthesized. Lower enzyme activities present at earlier stages of development would not be accurately determined even though these radiochemical methods of assay are the most sensitive presently available. As a result the project was abandoned.

Irving and Elliot (1969) have reached a similar conclusion in the investigation of ALA synthetase in guinea pig liver mitochondria. Although low enzyme activity appeared to be present when assayed by colorimetric techniques it could not be said with confidence to be significantly greater than zero using a more sensitive radiochemical assay.

Project III

N-acetyl serotonin transferase and monoamine oxidase activites in the pineal, cerebellum and cerebral hemispheres of the developing chick embryo.

I. Introduction

There is a considerable body of circumstantial evidence indicating that development and maturation of the immature brain is probably subject to indirect control by the pineal. Of particular significance is the fact that the embryonic chick pineal shows a marked secretory activity between day 15 and 19 prehatch (Campbell and Gibson, 1970). This secretory product has ready access into the third ventricle and cerebral spinal fluid prior to the development of the brain as measured by such criteria as myelination (Garrigan and Chargaff, 1963), electrical activity (Corner et al., 1967) and acetylcholinesterase activity (Burdick and Strittmatter, 1965). Melatonin has been demonstrated to be one pineal hormone and the development of some of the enzymes involved in its biochemical synthesis precede neural development in both the rat (Ellison et al., 1972; Snyder, 1968) and the chick (Wainwright, 1974a, 1974b). Moreover Relkin et al. (1973) have suggested the pineal may play a role in the control of myelination in the rat.

The long term objective, of which this work is a part, is an analysis of the interactions between the developing pineal gland and the developing cerebellum and cerebrum in organ culture with particular reference to the chick. These pineal brain interactions

probably involve regulation of the formation of specific enzymes. This thus necessitates the development of culture techniques such that enzyme ontogeny in organ culture reflects that in vivo.

Thus a first prerequisite is the study of the ontogeny in vivo of selected enzymes of the pineal and brain of developing chick embryos and young chicks. The development of HIOMT and hydroxytryptophan decarboxylase has already been reported (Wainwright, 1974a, 1974b). The main purpose of this work is to study the development of MAO and NAT. Serotonin has been suggested to be involved in brain development (Baker and Quay,1969)and MAO is involved in the breakdown of this compound. NAT is probably one of the key enzymes involved in the control of melatonin synthesis in the pineal. Both enzymes are also present in the cerebellum and cerebral hemispheres as well.

As the interaction of these tissues will be eventually studied in organ culture the in vitro development of MAO and NAT in pineals maintained for up to 6 days in organ culture was examined and contrasted with the identical in vivo period of 6 day growth. Norepinephrine causes a rapid rise in NAT activity of rat pineals in organ culture (Klein and Berg, 1970), and therefore the effects of this additive, as well as others, on the in vitro development of the chick pineal in organ culture were also examined.

Furthermore a circadian rhythm of NAT In the rat housed under diurnal lighting conditions has been established (Klein and Weller, 1970; Deguchi and Axelrod, 1972a). Therefore the activities of both NAT and MAO were examined over a 24 hour period in birds raised under various lighting conditions.

II. THE PINEAL GLAND

1a. Introduction (A General Description)

The pineal gland arises early in vertebrate development as an evagination of the third ventricle of the embryonic brain. The course of subsequent development of the gland varies markedly between different classes of animal and the mature glands of representative species show wide variation in structure and function.

In lower vertebrates, for example, the pineal consists of two separate structures, the epiphysis or pineal proper and the parapineal or parietal organ. In the lamprey these two components are almost identical, have separate stalks and are positioned in tandem, the parietal organ being the most superficial. The two organs differ considerably from each other in the lizard where the parapineal contains a well developed lens and retina and is situated beneath the scales of the head. This "third eye" is also present in the frog and is sometimes called the stirnorgan.

These pineal systems contain photosensory cells cytologically similar to cone cells of the retina (Collin, 1971), whose electrical activity is inhibited by light (Dodt and Heerd,1972; Miller and Wolbarsht, 1962). As pinealectomized tadpoles are unable to blanch in the dark, the pineal may play a role in adaptation to color background (Bagnera, 1960). Bagnera and Hadley (1970)

suggested that under conditions of darkness the pineal releases melatonin into the circulation where it causes aggregation of melanocytes within dermal melanophores resulting in a blanching of the skin. In higher vertebrates such as the mammal the pineal consists of a single glandular structure containing no photosensory cells (Kappers, 1960) and is not directly light sensitive. However it does obtain information about lighting conditions indirectly by way of the eyes. Although the pineal forms substantial amounts of melatonin it does not appear to have a role in the control of pigmentation and adaptation to color background. However early clinical observations of sexual precosity in patients with pineal tumors (Gutzeit, 1896; Del Rio Hortega, 1965) suggested a link between the pineal and gonadal function. Later investigations using both rats and hamsters indicated that the pineal is indeed antigonadotropic and probably acts via the pituitary gland by preventing production of pituitary gonadotropins (Kitay, 1967; Wurtman, 1967).

Although these antigonadotropic properties of the pineal are the most thoroughly investigated, other effects have been reported. It appears to inhibit other endocrine organs such as the thyroid and adrenal glands by suppressing the production of thyroxine (Sceporic, 1963; Houssay et al., 1966) and aldosterone (Kinson and Singher 1967) respectively. This drop in aldosterone production probably accounts for the insulin like effects (hypoglycemia, increase in hepatic and muscular glycogenesis) caused by inter-peritoneal injections of pineal extracts (Milcu et al., 1971).

The pineal also may modulate neural activity in the brain. Injection of melatonin into the blood has a sedative effect on the brains of animals (Barchas et al., 1967; Hishikawa et al., 1969) and has been used to combat the symptoms of epilepsy and Parkinsonism in man (Anton-Tay et al., 1971). Removal of the pineal, on the other hand, leads to an increase in both motor activity and electrical activity patterns of the brain (Nir et al., 1969). These effects are remarkably enhanced if the parathyroid gland is also removed as violent convulsion and death result (Reiter, 1972). Quay (1965a) has suggested the pineal may be involved in general brain homeostasis being involved in the maintenance of neural Na^+ and K^+ ion levels. An abnormal pineal may be involved in the human disease schizophrenia (Greiner, 1970; Hartley and Smith, 1973). Roles for the pineal in regulating embryological development of the brain (Relkin et al., 1973) and the duodenum (Owman, 1963, 1964a) have also been postulated. These known and suspected functions of the pineal have been reviewed by Mess (1968), Quay (1970) and Wolstenholme and Knight (1971), but it is probable we do not yet know all the roles of this intriguing organ.

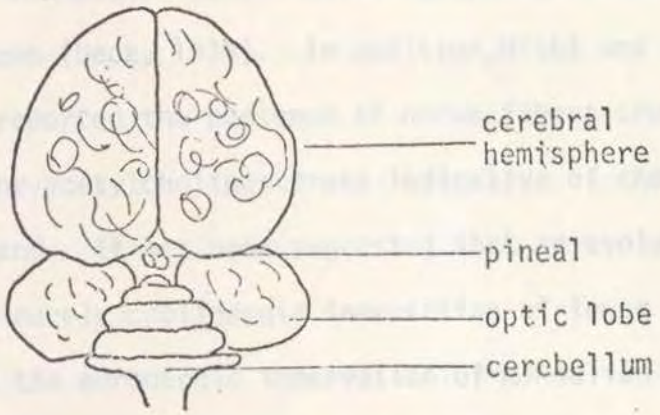
1b. The Avian Pineal

The avian pineal has characteristics of both lower and higher vertebrate types in that it is a producer of melatonin, is both directly and indirectly sensitive to light and has been reported to have an antigonadotropic function. It is a small conical

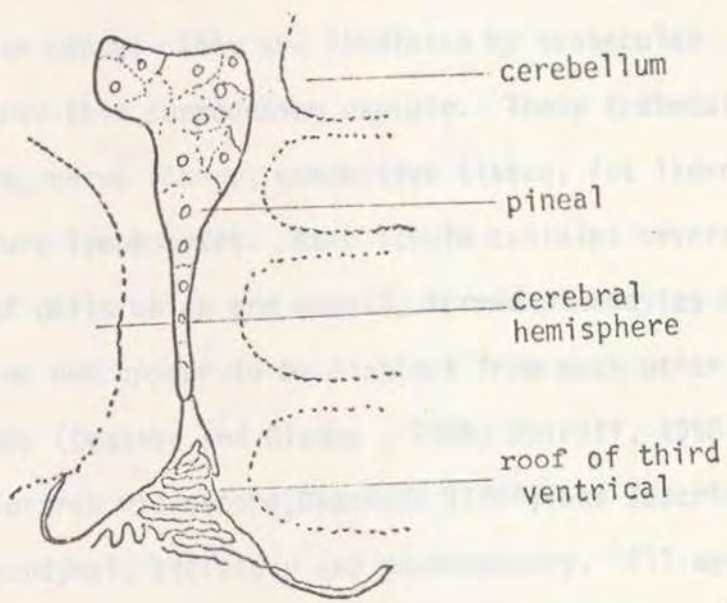
structure which lies in the triangular space between the cerebral hemispheres and the cerebellum, and extends dorsally towards the skull (Figure 30a). It consists of a distal glandular portion and a proximal stalk (Figure 30b) which is attached to the roof of the third ventricle. The size and shape varies greatly among species and is unrelated to body size.

In the domestic chicken the pineal is well supplied with blood (Beattie and Glenny , 1966). Although there is no information on the volume or velocity of this blood flow, the organ is metabolically very active and probably has as copious a vascular supply (Wight, 1971a) as has been described for the rat (Goldman and Wurtman, 1964). The circulation is striking in that the organ falls outside the restrictions imposed on the brain tissue by the "blood-brain barrier". Wight (1971a) showed clearly that the pineal is stained after systemic injection by such vital dyes as trypan blue, whereas the rest of the brain is not.

The avian pineal is richly innervated and may contain both adrenergic and cholinergic fibers. As is the case in the mammalian gland, the major innervation is by adrenergic fibers of the autonomic (sympathetic) system arising from the superior cervical ganglion, and removal of the two ganglia leads to complete sympathetic denervation of the pineal (Hedlund, 1970). Detailed analysis has shown this sympathetic system consists of an extensive network of fine neurites many of which are unmyelinated (Stammer, 1961; Fujie, 1968).



(a) The top view of an adult chicken brain showing the portion of the pineal with respect to other areas of the brain, Wight (1971) and Rogers (1960).



(b) Diagramatic representation of a longitudinal slice of the hen brain taken near the median sagittal plane, Oksche and Yaupel (1966).

Figure 30: The chick pineal; a diagramatic sketch.

Adrenergic fibers have also been observed in the pineal stalk (Kappers, 1965; Wight, 1971b), but it is not certain whether or not there is a functional connection to the third ventricle as has been described for the pigeon (Ueck, 1970). In addition, Wight and McKenzie (1970) have reported the presence of nerve fibers showing a positive reaction for acetylcholinesterase indicative of cholinergic innervation of the gland. It has been suggested that an evolutionary shift occurs from the purely cholinergic innervation of lower vertebrate pineals to the adrenergic innervation of mammalian pineals. The avian pineal contains both types of innervation and is morphologically intermediate between these two vertebrate groups.

It contains vesicles or rosettes of cells which are supported by thin intervesicular septa. They are lobulated by trabeculae arising from the rather thin surrounding capsule. These trabeculae contain blood vessels, nerve fibers, connective tissue, fat laden macrophages, and mature lymphocytes. Each lobule contains several vesicles or groups of cells which are usually termed pineocytes or pinealocytes. They do not appear to be distinct from each other under light microscopy (Beattie and Glenny , 1966; Spiroff, 1958). However using the electron microscope, Bischoff (1969) has described three cell types; ependymal, secretory and photosensory. All appear to be modifications of a basic pinealocyte consisting of a rounded base which contains the nucleus, a narrow neck and a rounded apex. The apical portion is in contact with the follicular lumen of the vesicle.

Ependymal cells contain numerous cilia and a few microvilli projecting into the follicular lumen. The cilia have a 9 + 2 arrangement of the microtubules. The secretory cells lack cilia but contain numerous membrane limited granules. The photosensory cells have possible synaptic contacts along their border and a modified cilia of a 9 + 0 arrangement projecting into the lumen. The lumen contains whorls and fragmented lamellar structures. This general description applies for most species of birds so far studied, including the chicken and the house sparrow (Oksche and Kirschstein, 1969; Oksche and Vaupl-von Harnack, 1966).

The photosensory cells lack the orderly arrangement of lamellae that characterize the pineal photoreceptor cells of amphibians and reptiles (Kappers, 1971; Collin, 1971). In addition, they do not exhibit electrical activity (Ralph and Dawson, 1968) and are therefore thought to be degenerate (Ralph, 1970).

In summary, comparison of the structure and properties of the avian pineal with those of the glands of both higher and lower vertebrates indicates there may have been an evolutionary modification of the function of this organ. The pineal of contemporary lower vertebrates remains primarily a sensory nervous element which responds directly to changes in environmental illumination. In contrast, the pineal of the higher vertebrates appears to act primarily as a neuro-endocrine gland and responds only indirectly to changes in illumination perceived by the retina, and has been termed a neuroendocrine transducer. The pineals of most birds are intermediate in structure

and exhibit both types of properties to at least some extent, while in yet other species the mature bird lacks any obvious pineal.

2. Biochemistry of the Pineal Gland

Biochemical studies of the pineal gland have been largely concerned with melatonin, biogenic amines and associated enzymes (Wurtman et al., 1968; Axelrod, 1970).

It has long been known (McCord and Allen, 1917) that the bovine pineal contains a substance which causes lightening of the skin pigmentation of amphibians. The active principle, N acetyl-5-methoxytryptamine was isolated and synthesized (Lerner et al., 1958) and named melatonin because of its effect on frog skin melanophores.

Pineocytes take up tryptophan from the blood and by successive hydroxylation, decarboxylation, acetylation and eventual methylation reactions melatonin is formed (Figure 31). Necessary catalysts, cofactors and donors have been shown to be present in the pineal. Details of the reactions have been recorded and reviewed for the rat (Klein and Berg, 1970) and the bird (Ralph, 1970).

Both serotonin and melatonin have been detected in the avian pineal (Quay, 1966; Hedlund and Ralph, 1967; Ralph et al. and Murphy, 1967). Serotonin is acetylated by serotonin-N-acetyltransferase (NAT) and the product then methylated by hydroxyindole-o-methyltransferase (HIOMT). The former enzyme is present in many tissues (Ellison et al., 1972). In contrast, the latter is restricted to the both the pineal and retina of the bird although activity is much

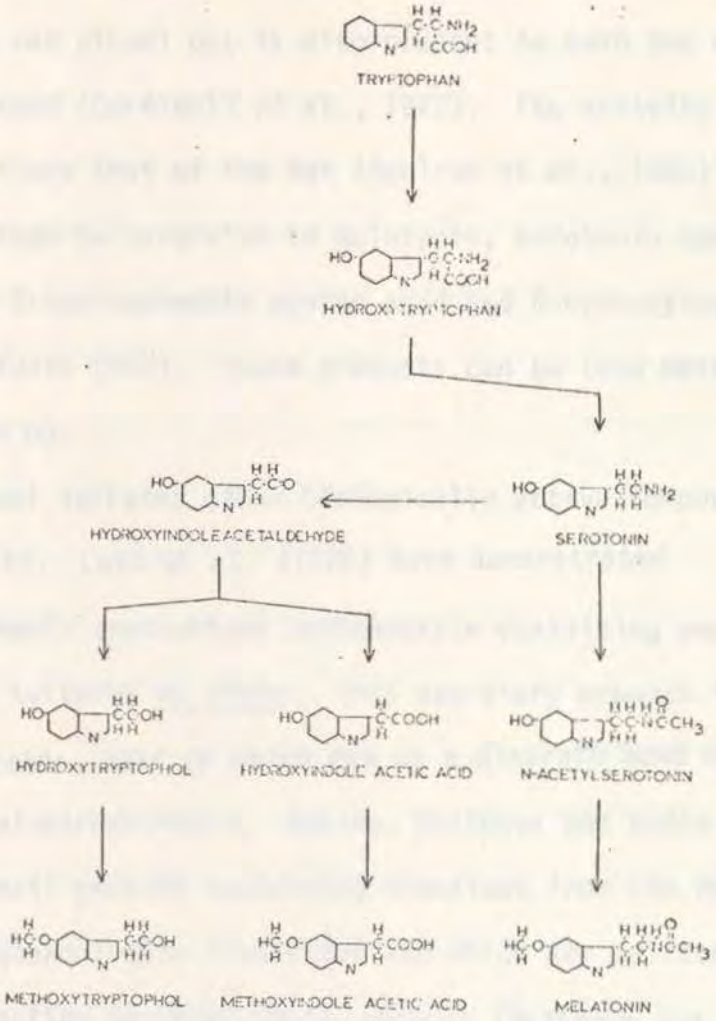


Figure 3I: Indole metabolism in the pineal gland. Tryptophan is converted to 5-hydroxy-tryptophan by tryptophan hydroxylase. Aromatic amino acid decarboxylase forms serotonin (5-hydroxytryptamine) from 5-hydroxytryptophan. Serotonin is converted to 5-hydroxyindole acetylaldehyde by monoamine oxidase. The unstable aldehyde is oxidized to 5-hydroxyindole acetic acid or reduced to 5-hydroxytryptophol. The alternative major pathway of serotonin metabolism is N-acetylation by N-acetyltransferase to form N-acetylserotonin. Hydroxyindole-O-methyl transferase converts serotonin, 5-hydroxyindole acetic acid, 5-hydroxytryptophol, and N-acetylserotonin to the following O-methylated derivatives: 5-methoxyserotonin, 5-methoxyindole acetic acid, 5-methoxytryptophol, and melatonin. From Klein and Weller (1970).

less in the retina (Quay, 1965). Similarly HIOMT shows greatest activity in the rat pineal but is also present in both the retina and harderian gland (Cardinali et al., 1972). Its activity in the chicken is 200 times that of the rat (Axelrod et al., 1964).

In addition to coversion to melatonin, serotonin can also be converted to 5-hydroxyindole acetic acid and 5-hydroxytoyptophol by monoamine oxidase (MAO). These products can be then methylated by HIOMT (Figure 31).

The pineal secretes other biologically active compounds besides melatonin. Lott et al. (1972) have demonstrated the release of newly synthesized carbohydrate containing peptides from rat pineal cultures in vitro. This secretory product is a mixture of proteins, half of which run as a discrete band on polyacrylamide gel electrophoresis. Benson, Matthews and Rodin (1972) have purified small peptide containing fractions from the rat pineal which have antigonadotropic properties and which are melatonin free. Proteolytic digestion destroys these effects (Matthews and Benson, 1973).

3. The Mammalian Pineal

(a) Embrylogical Development and Possible Functions at This Early Stage.

The growth of the embryonic rat pineal has been thoroughly investigated and reviewed by Kappers (1960). It develops as an evagination of the roof of the third ventricle on the 14th day of fetal life. By day sixteen the pineal has taken on a sac like appearance and is surrounded by a rich capillary network of blood

vessels. From day 17 to 19 the pinealocytes show evidence of pronounced secretory activity (Owman, 1961). As the pineal recess remains open to the third ventricle until after birth the secretory products have a direct access to the cerebral spinal fluid. By day 21 this secretory activity has almost ceased and the compact cellular structure of the pineal resembles that of the adult animal. After birth a link is maintained to the roof of the third ventricle by means of a thin closed stalk. At this stage the pineal contains essentially no nervous supply. However intrapineal nerves develop rapidly forming a network around vessels and parenchymal cells and by the third week have taken on a pattern similar to that of the adult (Hakanson et al., 1967). The ontogeny of some pineal enzymes including those involved in melatonin synthesis has been investigated. NAT activity is detectable four days prior to birth and reaches adult levels after three or four weeks (Ellison et al., 1972). This increase parallels development of the sympathetic nervous supply. The ontogeny of MAO also parallels this development. As early as four days of age a diurnal rhythm of NAT activity is evident. However this rhythm differs from the adult type in that it does not require a nervous supply and is not maintained under conditions of constant darkness (Machado et al., 1969). This is the first evidence that a mammalian pineal may be directly sensitive to light, at least at this early stage. HIOMT activity is low at birth and rises gradually to adult values after a period of five weeks. Thus although the pineal can produce N-acetylserotonin from serotonin

(b) Response to Variations in Lighting Conditions

Wurtman and Axelrod (1965) describe the pineal as a "biological clock" which synchronizes biological rhythms with changes in environmental conditions of light and darkness. It may be recalled that the pineal receives photic information from the lateral eyes. A sympathetic nervous pathway leading from the retina via the inferior optic tract to the superior cervical ganglia and hence to the pineal has been described (Axelrod and Wurtman, 1968; Eichler and Moore, 1971). The spontaneous activity of these nerves which occurs in the dark is blocked when light is perceived by the retina (Taylor and Wilson, 1970).

Under diurnal conditions of lighting there is a marked 24 hour or circadian rhythm in both the serotonin and melatonin content of the pineal gland. Serotonin shows a midday peak and a night time trough (Quay, 1963; Snyder and Axelrod, 1965a) whereas melatonin is 180^{o} out of phase with this rhythm (Figure 32) showing lowest values in the daytime and highest at night (Lynch, 1971).

Most of the pineal serotonin is stored within the parenchymal cells and sympathetic nerve endings and is unavailable to cellular enzymes. Onset of darkness triggers the release of serotonin where it then can either be lost into the circulation or enter into pineal cellular metabolism. MAO shows no change in activity over a 24 hour period (Snyder et al., 1965). Nevertheless because inter-peritoneal injections of β phenylisopropylhydrazine, a MAO inhibitor, blocks the nightly serotonin release MAO is partially responsible

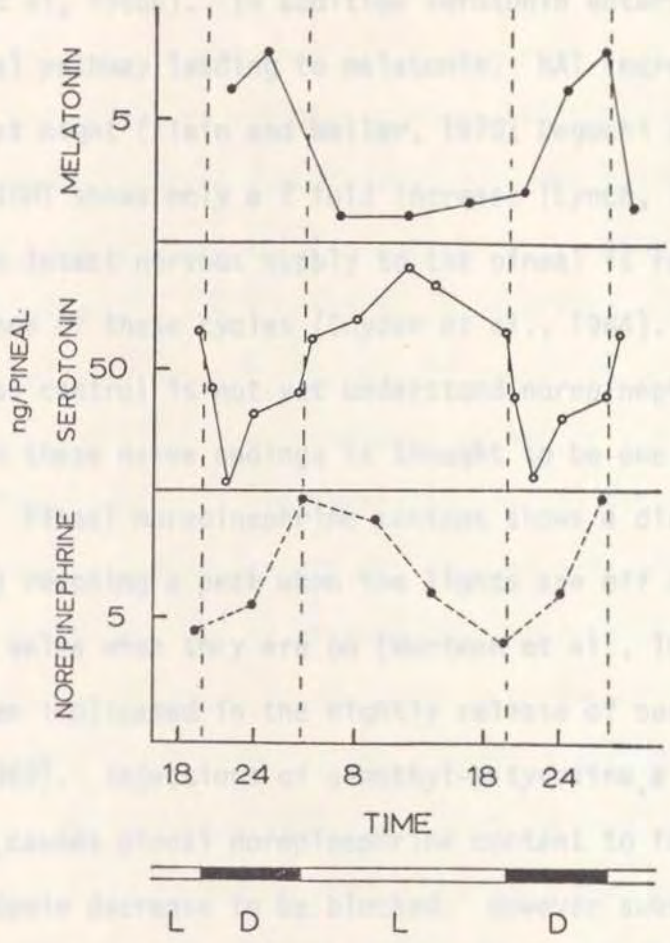


Figure 32: Circadian rhythms of serotonin, melatonin and norepinephrine content in the rat pineal. The rats were housed under diurnal lighting conditions (LD:14:10). The data is from Quay (1963), Wurtman et al. (1967) and Lynch (1971).

(Snyder et al, 1965a). In addition serotonin enters into the biochemical pathway leading to melatonin. NAT increases 30 fold in activity at night (Klein and Weller, 1970; Deguchi and Axelrod, 1972a) whereas HIOMT shows only a 2 fold increase (Lynch, 1971).

An intact nervous supply to the pineal is required for the maintainence of these cycles (Snyder et al., 1964). Although their biochemical control is not yet understood, norepinephrine which is present in these nerve endings is thought to be one of the factors involved. Pineal norepinephrine content shows a diurnal rhythm (Figure 32) reaching a peak when the lights are off and decreasing to a minimum value when they are on (Wurtman et al., 1967). Furthermore, it has been implicated in the nightly release of serotonin (Zweig and Axelrod, 1969). Injections of α-methyl-p-tyrosine, a tyrosine hydroxylase inhibitor, causes pineal norepinephrine content to fall and the night time serotonin decrease to be blocked. However subsequent injections of either noradrenaline or dopamine reverse these effects. Also norepinephrine has been implicated in the 30-fold increase in NAT activity by both *in vivo* and *in vitro* studies. In organ culture experiments addition of low concentrations (10^{-5} M) of noradrenaline causes a marked stimulation of /^{T4}C/-melatonin formation from either /^{T4}C/-tryptophan or /^{T4}C/-serotonin (Axelrod et al., 1969; Klein, 1969). This stimulation is due to a seven fold increase in NAT activity while HIOMT activity remains unchanged. As cycloheximide blocks the effect, the increase is probably due to *de novo* enzyme synthesis.

As both dibutyrl cyclic AMP and theophylline mimic the effect of norepinephrine Klein and Berg (1970) proposed that cyclic AMP mediates the effect of noradrenaline on melatonin synthesis. Norepinephrine injected subcutaneously causes a subsequent increase in NAT in vivo (Deguchi and Axelrod, 1972b). As propranol, a β adrenergic blocking agent, inhibited this effect whereas phenoxybenzamine, an α adrenergic blocker, did not it is probable that the pineal receptor sites for noradrenaline are of the former type.

These biological rhythms are normally synchronized to environmental lighting conditions. For example switching the diurnal lighting cycle 180^{o} out of phase (L:D → D:L) results in a corresponding phase shift in serotonin rhythm after a period of six days (Snyder et al., 1967). Disruption of the diurnal lighting also effects the cycle. NAT activity falls to a daytime level within 15 minutes if the lights are turned on during the dark portion of the lighting schedule (Deguchi and Axelrod, 1972a; Klein and Weller, 1972). In fact keeping animals under constant lighting abolishes all the cycles discussed so far (Snyder et al., 1965; Axelrod et al., 1965; Klein and Weller, 1970; Wurtman et al., 1967). If the lights are shut off early there is no subsequent increase in NAT activity (Binkley, Klein and Weller, 1973). Instead NAT increases at its regular time. Under conditions of constant darkness the rhythm of norepinephrine level is abolished; the level remaining midway between night time and day time values. Surprisingly cycles of serotonin

and NAT activity persist for as long as two weeks in blinded animals (Snyder et al., 1964) or in those maintained in continuous darkness (Snyder et al., 1965 ; Klein et al., 1970) and thus appear to be endogenous. However an NAT cycle was not evident in animals ten weeks after they were blinded (Klein et al., 1971). Instead NAT activities could be grouped as high (night time) or low (day time) values for each time of assay over a 24 hour period. Similarly no rhythm in pineal melatonin content was evident in rats 8 weeks after blinding; both high and low values being obtained for each period of assay (Reiter et al., 1971). Perhaps after blinding, enzyme activity is continuously low in some animals and high in others.

Klein offers another suggestion which could account for these erratic results. Animals exhibit various behavioral and metabolic circadian cycles (Aschoff, 1960; Luce, 1971), one well established example being motor activity (Quay, 1965c, 1970). In rats housed under diurnal lighting conditions this cycle of running activity peaks at night and all individual cycles are synchronized with respect to each other. Although the periods of these cycles remain constant for each animal maintained in continuous darkness they range between 24 and 36 hours within the group. Because of these slight differences in length of period, activity rhythms become asynchronous after a number of weeks. The monitoring of rhythms of melatonin content and NAT activity in the pineal gland requires one animal for each point assayed and it is assumed that the individual cycles are synchronized within the population. With diurnal lighting conditions

this assumption is probably valid. However, we have already noted that the rhythms are lost and only erratic values are obtained under conditions of constant darkness. These observations would result if the cycles acted in the same manner as that of running activity and became asynchronous in the absence of lighting cues (Klein et al., 1971). This suggestion is supported by the fact that the peak in pineal melatonin content coincided with the peak in running activity in rats 8 weeks after blinding (Reiter et al., 1971).

However, until these cycles can be individually monitored and their respective periods compared Klein's interpretation for the loss of rhythms remains only speculative.

Other biochemical and even morphological changes take place in the mammalian pineal when environmental lighting conditions are altered. Under constant illumination pineal weight decreases (Fiske et al., 1960) parenchymal cells become smaller in size (Roth et al., 1972) and HIOMT activity is decreased (Axelrod et al., 1965; Klein and Notides 1969). Blinding abolishes these effects (Wurtman et al., 1964). These results appear to be consistent with the fact that constant lighting blocks activity of the pineal nervous supply and in a sense the animal is environmentally pinealectomized. We have already discussed the fact that in darkness or in blinded animal pineal activity appears to be maintained.

(c) Some Possible Physiological Functions

An antigonadotropic effect of the pineal has been clearly established in mammals. Development of the gonads of immature rats

is enhanced after pinealectomy (Kincl and Benagiano, 1967; Roth, 1965) whereas interperitoneal injections of bovine pineal extracts inhibit gonadal development and overcome the effects of pinealectomy (Wurtman et al., 1959). Sorrentino (1968) has also shown that interperitoneal injections of melatonin leads to decreases in gonadal weights. Effects on mature rats are less marked but can be readily elicited under conditions of stress such as anosmia, starvation, or hormonal testosterone treatment (Reiter and Sorrentino, 1972). How these stresses potentiate the gonad inhibiting ability of the pineal is not understood.

Changes in environmental lighting which cause changes in pineal metabolism also effects gonadal development. Immature rats exposed to continual lighting show alterations similar to pinealectomized animals (Wurtman et al., 1961). As these combined conditions are not additive they may both be controlled by the same mechanism. Either injections of melatonin (Wurtman et al., 1965) or superior cervical ganglionectomy which block the pineals response to light also prevent these gonadal changes (Wurtman et al., 1964). The exposure of these animals to continuous darkness causes a moderate but significant retardation of sexual development and a similar decrease in the size of the adult gonads. These effects can be counteracted by pinealectomy (Reiter, 1968).

Although the testes of juvenile hamsters reach maturity at the same time regardless of the lighting conditions under which they are raised (Gaston and Menaker, 1967) adult gonads are extremely

sensitive to such environmental alterations. At least 12.5 hours of light per day are required for maintenance of both testicular weight and active sperm producing ability. If animals are housed under diurnal cycles of less light (e.g., L:D - 1:23) or none (D:D - 0:24) testes decrease in size and weight and a complete loss of spermogenesis results within a period of eight weeks, (Reiter(1968), Hoffman and Reiter, 1965). Blinding has an identical effect. HIOMT activity shows a four fold increase under these inhibitory conditions (Anton Tay and Wurtman, 1968). The importance of the pineal as a factor involved in this gonadal involution is underlined by the evidence that pinealectomy blocks this degeneration. It is interesting that this degeneration is not permanent,for after a period of 30 weeks testes regenerate spontaneously (Reiter, 1969, 1972). All the foregoing results were established with laboratory hamsters housed under carefully controlled environmental conditions. Reiter (1973a) found that hamsters housed outside for a year showed gonadal involution in the autumn when environmental lighting decreased and subsequently went into hibernation. Later in the spring (about 30 weeks later) the testes became enlarged and started producing sperm.

These observations suggest that the hamster pineal may act as an intermediary in the control of seasonal reproductive rhythms. Reiter (1973b) has described the following positive yearly cycle of events.

During the autumn of the year with decreasing photoperiod gonadal involution begins and the hamster enters hibernation. As spring approaches, the restraining influence which the pineal exerts is lifted and gonadal regeneration follows. When hamsters leave their burrows in the spring they are sexually competent and breed almost immediately. The young are born at a time of year optimal for their survival. The cycle reoccurs in the fall. As a result the pineal is implicated in a cycle which enables the hamster young to have the best chance of survival.

How the hamster distinguish between these long and short lighting schedules is not understood. Menaker (1971), however, has postulated a mechanism whereby photosensitive animals may measure these differences in lighting schedules. He suggests they do not use either the length of daylight or the length of darkness or even the ratio between the two as cues. Instead they distinguish between inductive and non inductive periods of daylength by assessing whether or not the light is present at a particular phase point on an endogenous circadian rhythm of sensitivity to its induction effect. Elliot et al. (1972) show evidence that such a circadian rhythm of light sensity in the hamster indeed exists. The position of light relative to this circadian rhythm is critical for a response such as gonadal involution to occur. In their experiment hamsters were subjected to various diurnal rhythms of lighting. They all received 6 hours of

light coupled with 18, 30, 42, or 54 hour periods of darkness (LD G:18, 6:30, 6:42, 6:54). Testis degeneration occurred only in the lighting cycles with periods of 24 and 48 hours. It may be recalled that over a 24 hour period the hamster requires at least 12.5 hours of light to maintain testis function. In the 36 and 54 hour diurnal periods the hamsters received light in the morning and also the following afternoon. These cues although spaced between periods of darkness were adequate to maintain gonadal size.

4.. AVIAN PINEAL

(a) Embryology and Possible Embryological Effects

The anlage of the pineal develops from a thickening of the epithelium in the roof of the third ventrical by the third day of incubation. The cells lining this evagination are ependymal in nature. In the five day embryo two cell types can be distinguished,the ependymocyte and the pineocyte. The ependymocyte seems to have a supportive function whereas the pineocyte is a secretory cell. These pineocytes are arranged in clusters and by day 7 these clusters have developed central lumen, have separated from the pineal recess and have formed primary vesicles. The amount of vesicular tissue increases and reaches a maximum by day 17 prehatch. The pineal is now a rather compact organ surrounded by a capsule. After day 17 the amount of vesicular tissue decreases and by 1 day post hatching only a few vesicles remain (Figure 33). By one month post hatching all vesicles have disappeared and the pineal gland develops the character of a compact lobed structure, (Campbell and Gibson, 1970; Spiroff, 1958). The secretory product of the embryonic pineal contains glycoproteins and sulfated polysaccharides formed from day 14 to 18 (Figure 34).

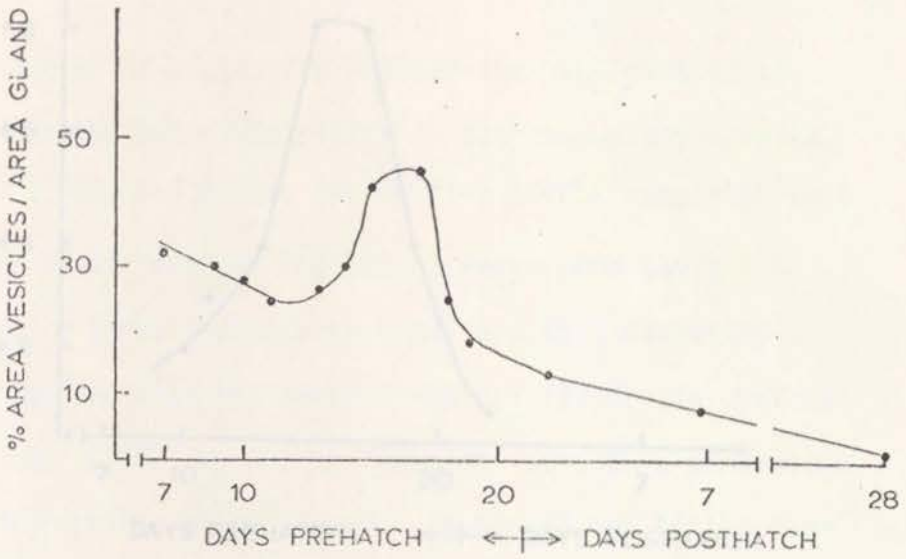


Figure 33: Vesicular portion of the chick pineal as a function of embryonic age (Campbell and Gibson, 1970).

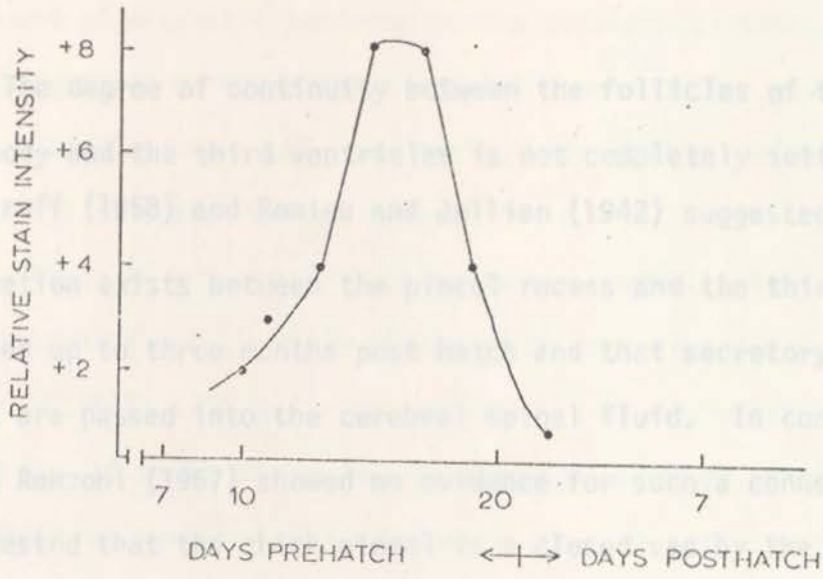


Figure 34: The comparative staining intensities of the luminal contents of the pineal vesicles as a function of embryonic age. These contents were stained with alcian blue, aldehyde fuchsin, periodic acid--Schiff reagent and metachromatic tuluidine blue (Campbell and Gibson, 1970).

The degree of continuity between the follicles of the pineal body and the third ventricles is not completely settled. Both Spiroff (1958) and Romieu and Jullien (1942) suggested that communication exists between the pineal recess and the third ventricles up to three months post hatch and that secretory products are passed into the cerebral spinal fluid. In contrast Quay and Renzoni (1967) showed no evidence for such a connection and suggested that the chick pineal is a closed sac by the time of hatch. In adult life the pathway is definitely closed and the rich pineal vasculature is a more likely route for any secretory efflux.

Embryonic roles for the embryonic chick pineal have not as yet been clearly elucidated. No effects upon endocrine function have been observed following pinealectomy of young chicks. Stahlsberg (1965) pinealectomized 285 6 day embryonic white leghorn chicks. Over one-third hatched and survived until they were sacrificed at 18 and 63 days of age. The low survival rate was due to the trauma of the operation since operated controls had the same low survival rate. All the pineals regenerated to some extent (from .01% normal to nearly normal size). No attempt was made to measure

the amount of enzymatic activity in the regenerated tissues. Stahlsberg found no correlation between the amount of remaining pineal tissue and survival, sex ratio, body size or weight or activity of endocrine or sex organs.

There is a great deal of suggestive evidence that the embryonic chick pineal may be involved in brain development (Wainwright 1974a; 1974b). The secretory activity which occurs between day 14 to 18 of the embryo precedes onset of chick neural development as measured by sustained electrical activity (Corner et al., 1967), by myelination (Garrigan and Chargaff, 1963; Shaikh, 1973) or by increase in key enzymes associated with activity of the central nervous system such as acetyl cholinesterase (Burdick and Strittmatter, 1965). It may be recalled that the rat pineal has recently been shown to have a possible role in the control of myelination in the brain (Relkin et al., 1973).

(b) Response to Variations in Lighting Conditions

The avian pineal has not been as extensively investigated as that of the rat. However, similar circadian rhythms of pineal metabolites and enzymes have been reported. In a 12:12 light dark cycle serotonin is at a maximum near the beginning of the light phase and at a minimum near the middle of the dark phase in both the

pigeon (Quay, 1966) and the Japanese Quail (Hedlund et al., 1971). Melatonin content, in contrast, is highest during the dark phase (Ralph et al., 1967; Lynch, 1971). Shifting the light cycle 180^{o} out of phase results in a similar phase shift of melatonin rhythm. The melatonin cycle was evident in chick as early as two days of age (Lynch, 1971). The slight increase of HIOMT at night (Pelham and Ralph, 1972) has not consistently been reported (Binkley et al., 1973; Oishi and Lauber, 1973) NAT shows a three to thirty fold increase at night (Backstrom et al., 1972; Binkley et al., 1973) and is thus partially responsible for the serotonin and melatonin rhythms.

In direct contrast to the effect on the rat pineal continuous lighting leads to an increase in both hen pineal weight and HIOMT activity (Axelrod et al., 1964). As this increase is neither blocked by blinding nor by severing of the superior cervical ganglia (Lauber et al., 1968), the avian pineal may be directly light sensitive. Direct illumination also increases HIOMT activity as well as the conversion of serotonin into N acetylserotonin and melatonin in duck pineal explants (Rosner et al., 1971, 1972). However morphological studies suggest that pineal photo receptor cells are degenerate (Kappers, 1971) and Ralph and Dawson (1968) have failed to show any changes in pineal electrical activity relating to environmental lighting differences. This controversy concerning

the direct photosensitivity of the avian pineal has been discussed by Ralph (1970) and still remains unresolved.

(c) Some Possible Physiological Functions

A great majority of avian species undergo an annual reproductive cycle in which the male gonads increase in size and mature in the early spring in preparation for the ensuing breeding season (Wolfson, 1966; Farner, 1964, 1970). The retina is not involved in this photoperiodic response in either the duck (Benoit, 1964) or the sparrow (Menaker and Keats, 1968; Underwood and Menaker, 1970). Although the pineal responds to variations in lighting regimes and its HIOMT activity shows an annual cycle the mirror image of the testicular cycle (Barfuss and Ellis, 1971) its role in the annual testicular cycle has not as of yet been clearly elucidated. It is probably not the extraretinal photo receptor mediating the effects on the testis (Farner, 1970; Menaker, 1971). Barfuss and Ellis (1971) indicated that the increase in sparrow testicular weight occurs prior to the decrease in HIOMT activity. Moreover, birds housed under red lighting conditions showed an increase in pineal HIOMT activity but no decrease in testicular weight. In addition pinealectomy had no detectable influence on the photoperiodic control of testicular growth (Oksche et al., 1972).

Nevertheless, the pineal of the bird appears to effect gonadal development. Shellabarger (1953), and Kitay and Altschule (1954)

list the relevant publications, the majority of which claim that hypertrophy of the fowl's gonads follows pinealectomy and that gonadal hypotrophy follows the injection of pineal extracts. Shellabarger (1950, 1952, 1953) studied chicks pinealectomized at one day of age. At day 20 the testes were atrophied whereas between day 40 to 60 after the operation the testes were hypertrophied. Singh and Turner (1967) have demonstrated that injections of melatonin decrease the weight of testes and ovaries of developing chicks. Investigators have not always been able to show an effect of the pineal on the gonads (Stahlsberg, 1965; Ralph, 1970). This may be attributed to technical imperfections including damage to other parts of the nervous system during pinealectomy and also partly due to a multiplicity of factors effecting the reproductive system of which the pineal is only one.

One interesting approach which has clearly demonstrated that the avian pineal can exert an antigonadal effect upon the embryonic testes has been devised by Moskowska (1958). The intact embryonic pineal maintained in organ culture continues to develop with essentially normal morphology, except for an excessive deposition of melanin granules (Vidmar, 1953). The test system consisted of a mixed organ culture in which development of the embryonic testis was stimulated by an adjacent embryonic hypophysis. Inclusion of embryonic pineals antagonized this stimulatory effect

of the hypophysis on the testis (Moskowska , 1956). As a modification of this approach Moskowska (1958) has also examined the effects of pineal grafts on guinea pig ovary. Permanent estrus due to lack of gonadotropic luteinizing hormone results. In contrast adult hen pineals failed to show this effect.

There are several lines of suggestive evidence that the pineal may play a role in regulating the locomotor behavior of birds. Under normal conditions birds exhibit a diurnal cycle of activity parallel with the lighting cycle. Maximum activity coincides with the light phase and birds perch or roost in the dark (Menaker, 1968). Disruption of the light cycle markedly effects the cycle of behavior. In continuous lighting the activity rhythm is abolished. Birds blinded or kept in constant darkness maintain a circadian rhythm of activity. However as there are no lighting cues the cycle has a period of slightly more than 24 hours (Figure 35). As a result the beginning and end of the activity cycle shifts by the amount of this increase daily. Pinealectomized birds will still entrain to light cycles and show circadian rhythms of locomotor activity. However now in the absence of lighting cues (e.g., constant darkness) the cycle is abolished. It has been suggested that the pineal may be a biological clock (Gaston and Menaker, 1968) and it is involved in the maintenance of the endogenous rhythm of locomotor activity. Indeed rhythms of both serum melatonin content (Pelham et al., 197) and pineal melatonin

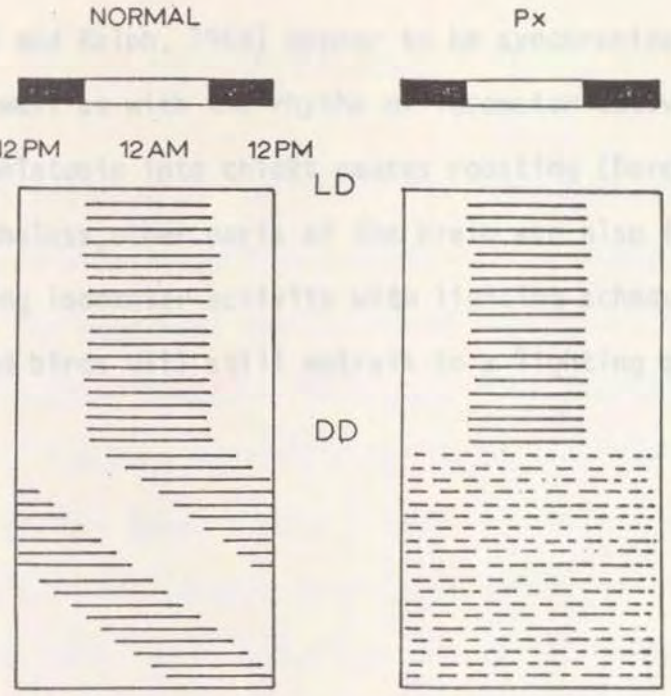


Figure 35: Activity rhythms of normal and pinealectomized house sparrows maintained under various conditions of lighting. Each line on the chart represents a period of 24 hours and the darkened portions mark perch hopping activity (Menaker, 1971).

content (Lynch and Ralph, 1968) appear to be synchronized with each other as well as with the rhythm of locomotor activity. Injection of melatonin into chicks causes roosting (Barchas et al., 1967). Nevertheless, other parts of the brain are also involved in synchronizing locomotor activity with lighting schedules as pinealectomized birds will still entrain to a lighting schedule.

III. Methods

1. Incubation and raising of the chicks

Fertilized eggs of the Shaver "Starcross No. 288" line of White Leghorn were obtained from Lone Pine Farm Ltd., Berwick, Nova Scotia. The eggs were incubated and hatched at 37° C in Humidaire incubators (Humidaire Incubation Company, New Madison, Ohio, U.S.A.). The eggs hatched after 20-22 days of incubation and the day of hatch was taken as day 0 for the ontogeny studies.

Three sets of lighting conditions were used in the rearing of the chicks after hatching, namely (a) maintained in constant lighting (LL, 24:0), (b) maintained in constant darkness (D:D, 0:24) and (c) maintained in the dark for three days and then transferred to a diurnal cycle of 14 hours light -10 hours darkness (L:D,14:10).

In the studies of the diurnal rhythm of NAT, day old chicks were purchased and raised under the three lighting conditions previously described.

2. Assay for NAT Activity

In the studies of the ontogeny of NAT, chicks were decapitated between 10:00 and 11:00, to minimize the effects of any diurnal cycle in the levels of enzyme activity. For studies of the diurnal cycle of NAT activity chicks were decapitated at the appropriate times. The pineal, cerebral hemispheres and cerebellum were rapidly removed and placed in a dish of ice cold 0.9% (w/v) saline. As

rapidly as possible the tissues were blotted lightly, weighed and homogenized in 0.1 M phosphate buffer (pH 6.8) in a Duall tissue cylinder (Kontes Glass Co. Vineland, New Jersey). The pineal was homogenized in 0.33-0.50 ml of buffer in order to give a concentration of 6-8 mg (wet weight)/ml, whereas the cerebral hemispheres and cerebellum were homogenized at concentrations of 200 mg/ml and subsequently diluted to 50 mg/ml and 12.5 mg/ml respectively.

Aliquots of the homogenate were assayed for NAT activity as described by Klein and Notides (1969), except that the products were separated by chromatography in only one dimension. All tissues were assayed at three concentrations (to measure linearity of response) at 37° C in a total volume of 50 λ 0.1 M phosphate buffer pH 6.8 containing 10 nM acetyl coenzyme A and 20 nM of /2-^{14}C/ serotonin binoxalate (New England Nuclear, 26.7 μC/μ mole). After 10 minutes the reaction was stopped by the addition of 50 λ 95% (v/v) ethanol and 1% ascorbic acid in 0.1 N HCl (2:1) containing N acetylserotonin, melatonin and 5-hydroxyindole acetic acid all at a concentration of 5 nM. The mixture (60 λ) was then spotted onto a 0.25 mm Merck-type silica gel plate (5 x 20 cm, silica Gel F-254, Brinkman Instrument Company) and developed by ascending chromatography. To increase resolution the plates were rechromatographed three times. Chromatographs were developed in the dark in chloroform; methanol; acetic acid (93:7:1) the time taken for the four runs was about six hours. For spotting and drying between runs the plates were kept in an atmosphere of 95% N_2. When the two

dimensional chromatographic system was used for comparative purposes, plates were turned 90° and chromatographed once in ethyl acetate. The separated metabolites of serotonin were visualized under ultraviolet light, outlined in pencil and scraped into counting vials. To each vial was added 1 ml of toluene , 5 mls of scintillation fluid (Toluene - POPOP - POP cocktail) and the samples were counted by standard liquid scintillation techniques.

3. Assay for MAO Activity

The labelled product HIAA produced under assay conditions already described was scraped from the silica gel plate and used to measure the MAO activity (Hakanson and Owman, 1965).

4. Protein and DNA

The protein content of the various homogenates was obtained by the method of Lowry et al. (1951) using bovine serum albumin as standard.

DNA content of cerebral hemispheres and cerebellum was measured by the method of Abraham et al. (1972) using DNA of M. Luteus (Miles Laboratory, Inc.) as standard.

5. Pineal Organ Culture

Pineals were cultured in 1 ml of media 199 (Grand Island Biological Company) at 38° C as described by Rosner et al. (1971).

Depending on the length of time of incubation, media were changed every two days. Gas chambers were designed to maintain a 5% CO_2 atmosphere (New, 1966) and were gassed with 95% O_2, 5% CO_2 prior to incubation. Three pineals were cultured together in culture vessels consisting of a 2.5 cm watch glass fitted with a stainless steel screen (.33 mm mesh) set in a 4.0 cm watch glass and placed in a 10 cm petri dish moist chamber. Each gas chamber could hold up to four petri dishes.

At the time of assay the pineals were rinsed in 0.9% (w/v) saline, weighed and homogenized in 0.1 M phosphate pH 6.8, and enzyme activity was measured as described previously.

IV. Results

1. Modification of Assay Procedure for NAT Activity and Assay of MAO Activity

Crude homogenates of brain and pineal convert serotonin into several metabolites including hydroxytryptophol, hydroxyindole acetic acid and N acetyl serotonin. These may be methylated to form the corresponding methoxyindoles. In the conventional assay of Klein et al. (1969) these various products are resolved by TLC in 2 dimensions (Figure 3G). In studies where /^{14}C/-serotonin of the highest available specific activity is used as substrate the limit of sensitivity of the assay is determined by the maximum size

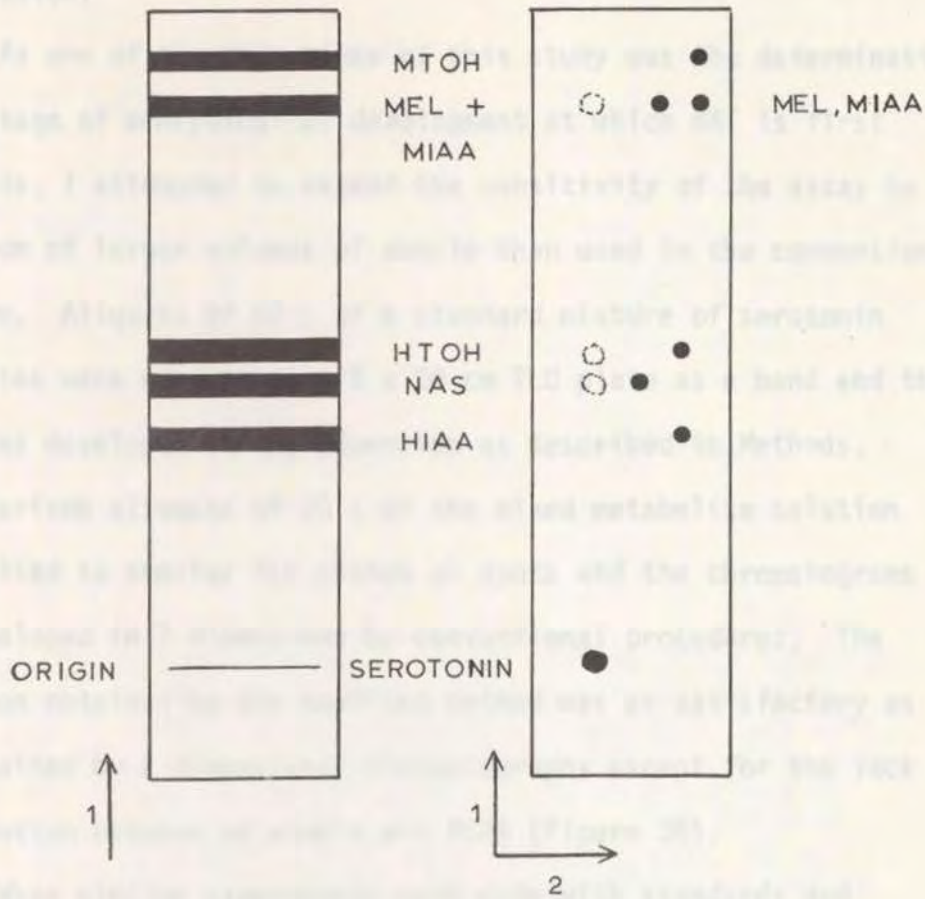


Figure 36: Separation of serotonin metabolites by one and two dimensional chromatography. Standards were spotted on the plates and chromatographed 4X in chloroform:methanol:acetic acid, 97:3:1. The second plate was turned 90° and chromatographed once in ethyl acetate. Samples were outlined in pencil under u.v. light.

of sample which may be applied to the chromatogram without sacrifice of resolution.

As one of the objectives of this study was the determination of the stage of embryological development at which NAT is first detectable, I attempted to extend the sensitivity of the assay by resolution of larger volumes of sample than used in the conventional procedure. Aliquots of 60 λ of a standard mixture of serotonin metabolites were applied to a 5 x 20 cm TLC plate as a band and the sample was developed in one dimension as described in Methods. For comparison aliquots of 20 λ of the mixed metabolite solution were applied to similar TLC plates as spots and the chromatograms were developed in 2 dimensions by conventional procedures. The resolution obtained by the modified method was as satisfactory as that obtained by 2 dimensional chromatography except for the lack of resolution between melatonin and MIAA (Figure 36).

When similar experiments were made with standards and tissues incubated as discussed in Methods, an additional radioactive product was observed on chromatograms prepared by the modified procedure. This unidentified product was formed by all tissues studied and contained levels of radioactivity as high or greater than those of HIAA or NAS. It travelled slightly behind melatonin on the plate (Figure 37). Furthermore in these experiments the level of radioactivity recovered in the melatonin band was up to 30%

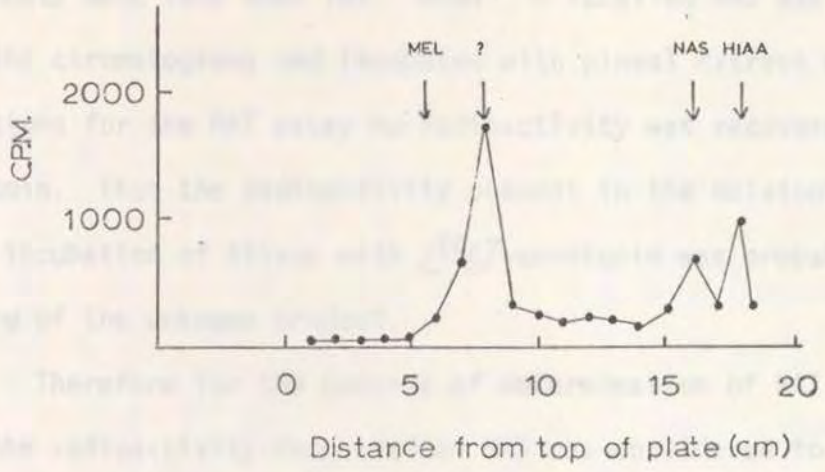


Figure 37: A chromatographic profile of the /^{14}C/ labelled products of /^{14}C/ serotonin formed by a chick pineal (2 day) homogenate. Strips (1 cm) were scraped from the plate into counting vials and radioactivity was determined as described in methods. The positions of MEL, NAS and HIAA standards (visualized in u.v. light), as well as the position of the unidentified metabolite are indicated by arrows.

of that recovered in NAS whereas in a two dimensional separation the counts were less than 10%. When ^{14}C labelled NAS was recovered from the chromatograms and incubated with pineal extract under conditions for the NAT assay no radioactivity was recovered as melatonin. Thus the radioactivity present in the melatonin spot after incubation of tissue with /^{14}C/-serotonin was probably due to tailing of the unknown product.

Therefore for the purpose of determination of NAT activity only the radioactivity recovered as NAS was considered to be the product formed. Activities measured by this method were linear with time for up to 15 minutes of incubation and with 3 concentrations of extract for the three tissues studied (Fig. 38, 39).

MAO activity was linear with concentration of extract and showed linearity with time up to a period of 15 minutes for all tissues studied (Fig. 40, 41).

2. Circadian Rhythm of NAT

Over a 24 hour period the pineal activity showed a five fold variation under diurnal (16:8) lighting conditions. As soon as the lights went out NAT activity began to increase until it reached a peak after 5 hours of darkness, and declined before the lights went on in the morning (Figure 42). This cycle was evident in birds 16, 18 and 20 days post hatch. Neither the cerebellum nor the cerebral hemisphere NAT had this diurnal rhythm of activity

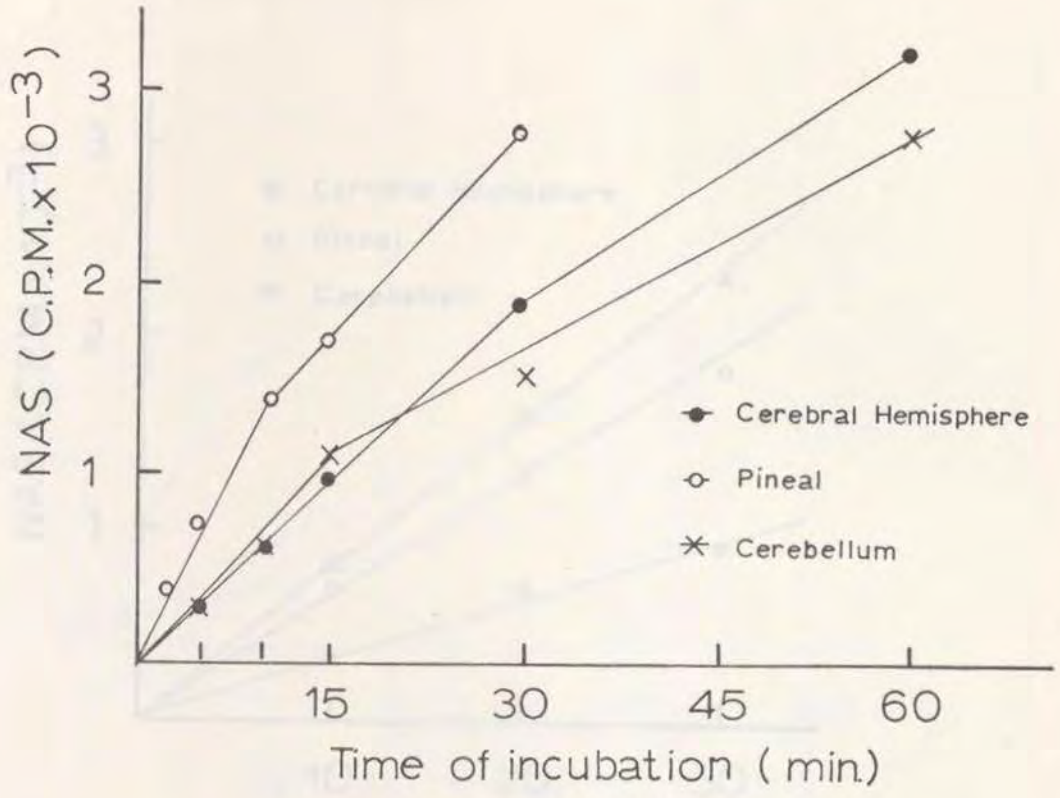


Figure 38: The linearity of serotonin N acetyl transferase activity with time of incubation. Volumes of 20 λ homogenate (pineal, 6.00 mg/ml; cerebellum 25 mg/ml; cerebral hemisphere 6.25 mg/ml) were incubated as described in methods for the time periods indicated. Tissue was taken from 4 day old chicks.

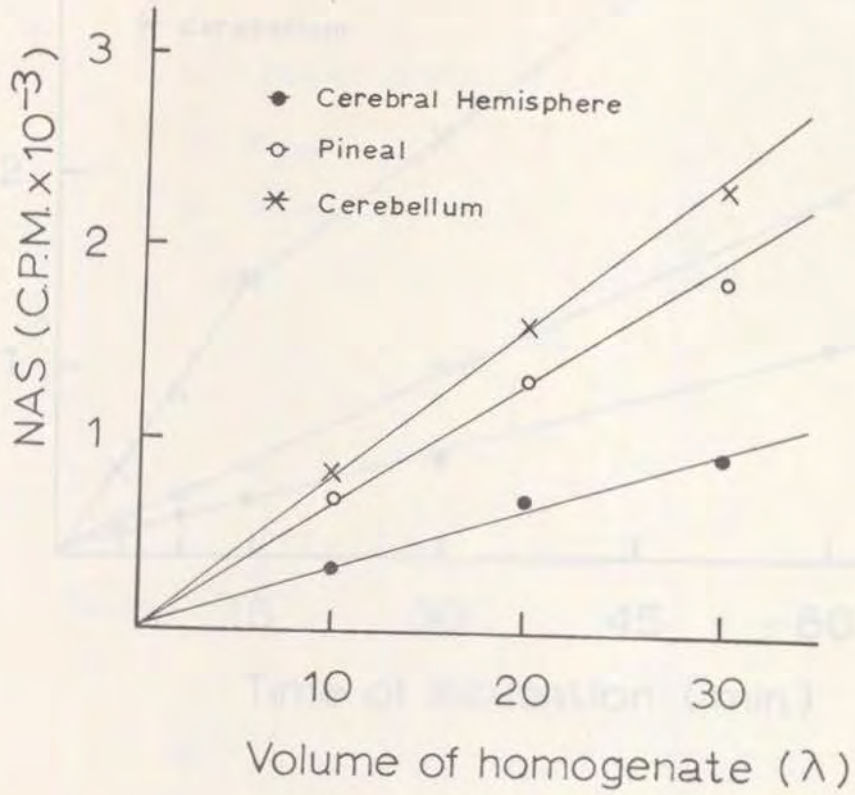


Figure 39: The linearity of serotonin N acetyl transferase activity with concentration of enzyme. Tissues (pineal, 6.00 mg/ml; cerebellum 25 mg/ml; cerebral hemisphere, 6.25 mg/ml) were incubated 10 minutes as described in methods. Tissues were taken from 4 day old chicks.

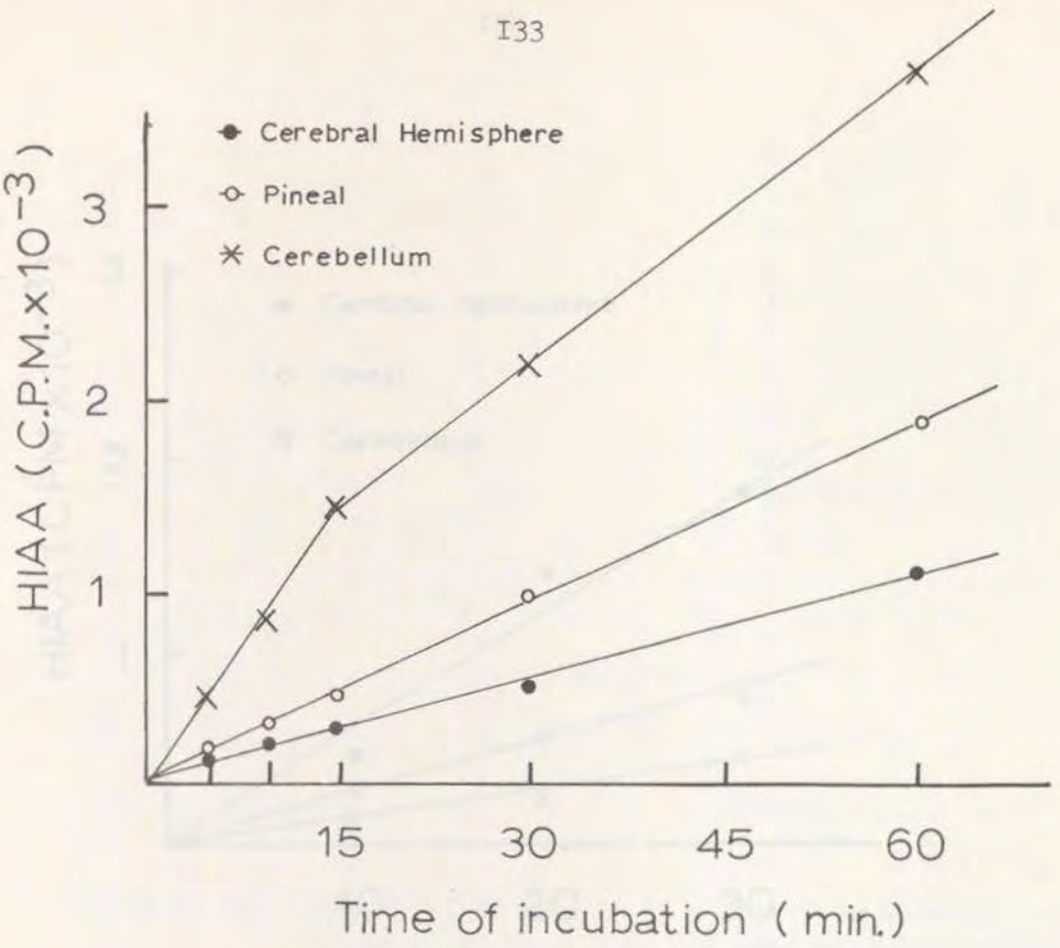


Figure 40: The linearity of monoamine oxidase activity with time of incubation. Volumes of 20 λ homogenate (pineal, 6.00 mg/ml; cerebellum 25 mg/ml; cerebral hemisphere 6.25 mg/ml) were incubated as described in methods for the time periods indicated. Tissue was taken from 4 day old chicks.

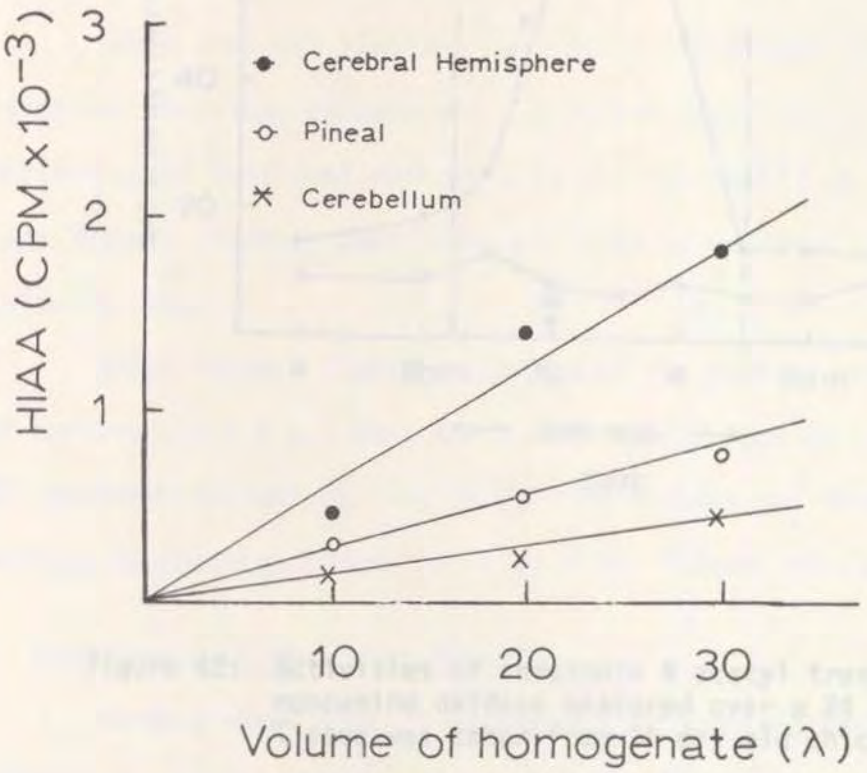


Figure 41: The linearity of monoamine oxidase activity with concentration of enzyme. Tissues (pineal, 6.00 mg/ml; cerebellum 25 mg/ml; cerebral hemisphere, 6.25 mg/ml) were incubated for 10 minutes as described in methods. Tissues were taken from 4 day old chicks.

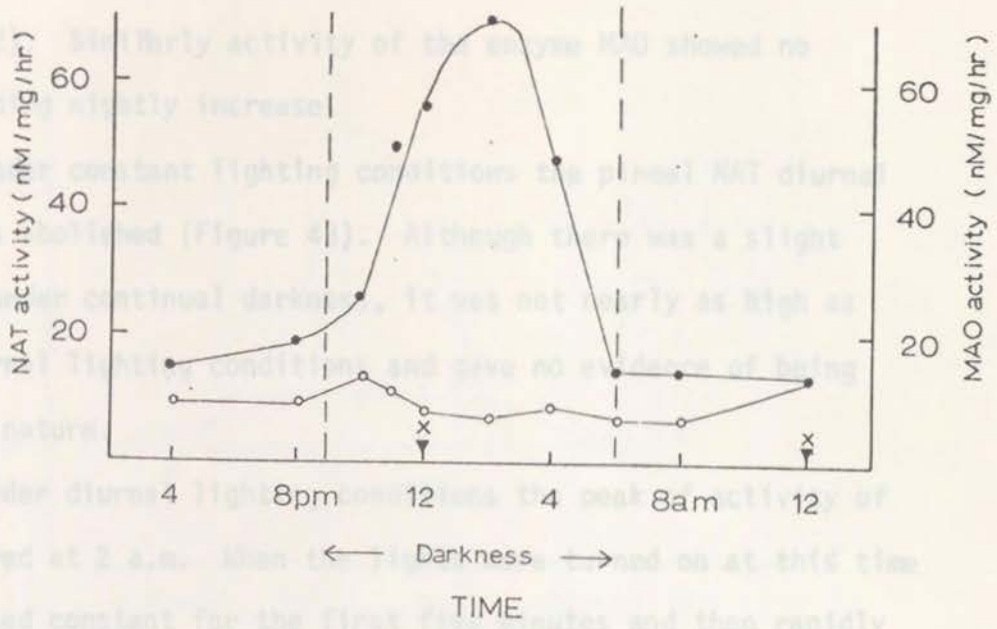


Figure 42: Activities of serotonin N acetyl transferase and monoamine oxidase measured over a 24 hour period. Tissue was taken from 16 day old chicks.

- • NAT activity of the pineal.
- × NAT activity of the cerebellum.
- ▼ NAT activity of the cerebral hemisphere.
- o MAO activity of the pineal.

(Figure 42). Similarly activity of the enzyme MAO showed no corresponding nightly increase.

Under constant lighting conditions the pineal NAT diurnal rhythm was abolished (Figure 43). Although there was a slight increase under continual darkness, it was not nearly as high as under diurnal lighting conditions and gave no evidence of being cyclic in nature.

Under diurnal lighting conditions the peak of activity of NAT occurred at 2 a.m. When the lights were turned on at this time NAT remained constant for the first five minutes and then rapidly declined approaching a daytime value after fifteen minutes (Fig. 44).

3. Development of the Chick Pineal

Various conditions of environmental lighting are known to effect avian pineal weight, HIOMT activity (Axelrod et al., 1964) and NAT activity (Ellison et al., 1972; Klein et al., 1972) (Fig. 42, 43). Therefore the development of the chick pineal after hatch was studied in birds raised under three programs of lighting; namely, continual lighting (L:L, 24:0), continual darkness (D:D, 0:24) and diurnal lighting (L:D, 14:10).

As there were no significant differences in pineal weight increases from hatch until day 18 the data for birds raised under the various lighting schedules were pooled (Figure 45). The weight of the embryonic pineal doubled between the 13th and 15th day of incubation and then remained constant until 4 days post hatch.

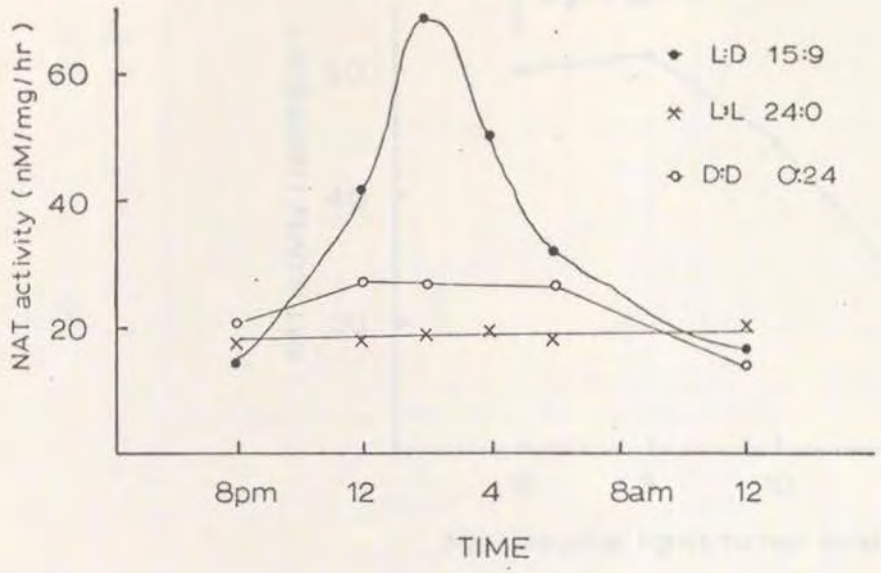


Figure 43: Effect of different lighting conditions on the circadian rhythm of pineal serotonin N acetyl transferase. Day old chicks were raised under the lighting schedules indicated and sacrificed at 18 days of age. The procedure for determining NAT activity is described in methods. Protein was determined by the method of Lowry et al. (1951).

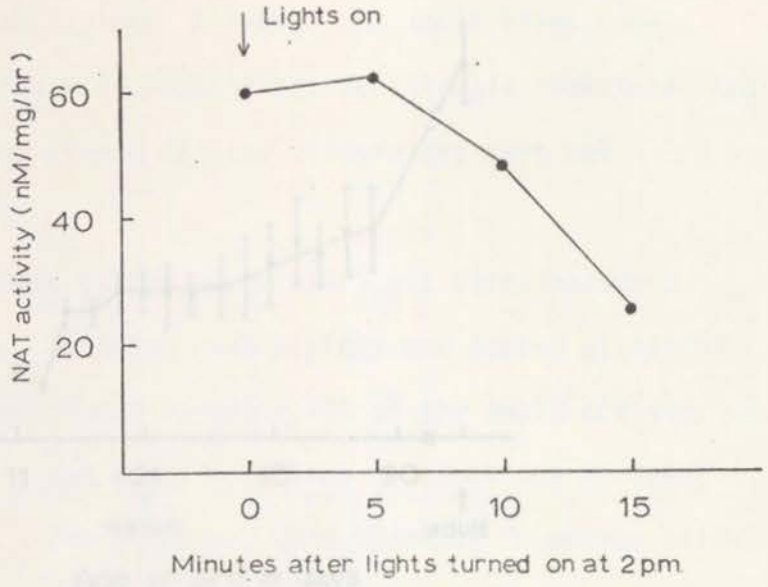


Figure 44: Effect of light on the night time increase of pineal N acetyl transferase activity. Chicks were raised under diurnal lighting conditions (15:9) and sacrificed at 16 days of age. At 2:00 A.M. the lights were turned on and activities of pineal N acetyl transferase were measured as described in methods.

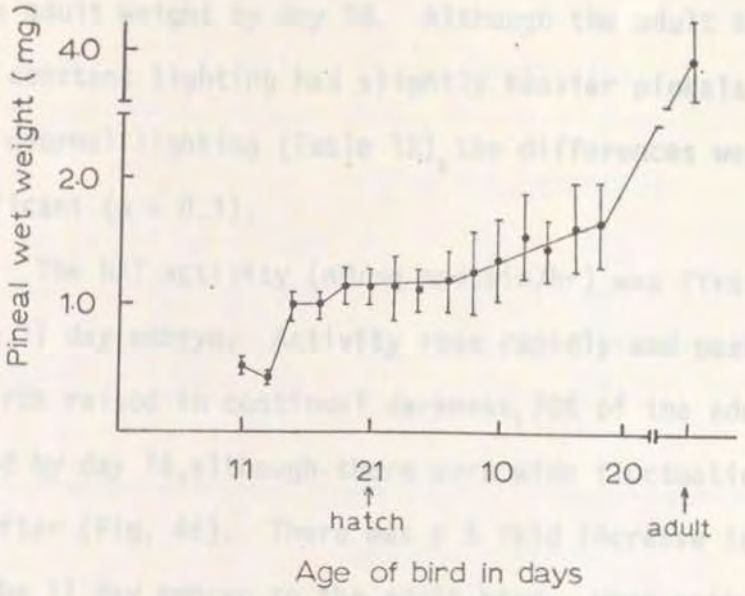


Figure 45: The wet weight of the chick pineal as a function of age. Pineals were taken from chicks at the age indicated and weighed immediately. The bars indicate 1 standard deviation and the results are an average of 9 or more determinations. As there were no significant differences between the weights, pineals for birds raised under different lighting conditions (L:L, L:D, D:D) data were pooled.

Thereafter the weight increased gradually and had reached 40% of the adult weight by day 18. Although the adult birds housed under constant lighting had slightly heavier pineals than those kept under diurnal lighting (Table 12), the differences were not significant ($p > 0.1$).

The NAT activity (nM/mg protein/hr) was first measured in the 11 day embryo. Activity rose rapidly and peaked at hatch. For birds raised in continual darkness, 70% of the adult activity was reached by day 18, although there were wide fluctuations in level thereafter (Fig. 46). There was a 5 fold increase in enzyme activity from the 11 day embryo to the adult bird. When activity was expressed on a mg wet weight basis essentially the same increase was observed. As the pineal was increasing in weight total NAT activity per pineal increased 48 fold from the 11 day embryo to the adult bird. NAT activity showed the greatest rate of increase between days 13 to 17, however.

Activities for birds raised under diurnal lighting (Figure 47) increased gradually from day 6 to 18 post hatch and showed less fluctuation. Under constant lighting conditions NAT activity increased more rapidly after hatch when compared to birds raised under the two previously described lighting conditions (Figure 48). Adult activity was reached by day 8. Eight and ten day levels of activity were significantly higher ($p < .05$) than

TABLE 12

Tissue		Pineal	Cerebellum	Cerebral Hemisphere
		nM/mg wet weight/hr		
NAT	LL	1.52 ± .290	.199 ± .018	.291 ± .036
	LD	1.49 ± .010	.245 ± .020	.391 ± .079
MAO	LL	1.00 ± .24	.488 ± .024	.367 ± .076
	LD	1.13 ± .10	.533 ± .074	.314 ± .059
		nM/mg protein/hr		
NAT	LL	17.4 ± 3.5	2.73 ± .13	5.70 ± .00
	LD	17.1 ± 1.50	2.45 ± .31	7.25 ± 1.20
MAO	LL	11.5 ± 3.3	6.70 ± .40	5.10 ± 1.5
	LD	12.7 ± 1.04	7.4 ± 1.30	5.80 ± 1.5
		nM/pineal/hr	nM/mg DNA/hr	nM/mg DNA/hr
NAT	LL	6.85 ± .46	45.3 ± 3.3	291 ± 8.1
	LD	6.83 ± 1.01	54.3 ± 4.9	305 ± 43
MAO	LL	4.21 ± .87	107 ± 12.3	287 ± 92
	LD	4.72 ± .63	115 ± 22	255 ± 67
		pineal wet weight		
	LL	4.30 ± 0.50		
	LD	3.65 ± 0.45		

Activities of NAT and MAO in the pineal, cerebellum and cerebral hemispheres in the adult hen. Values are the average of three determinations ± standard deviation. See methods for description of enzyme assay.

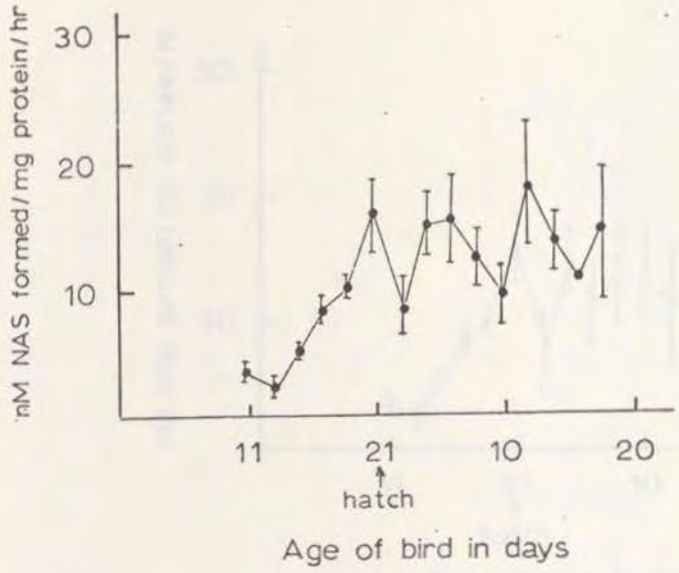


Figure 46: The ontogeny of serotonin N acetyl transferase of chicks raised in the dark. Pineals were taken from chicks or chick embryos at the age indicated and enzyme activity was measured as described in methods. The bars indicate the standard deviation and each point is the average of 3 or more determinations.

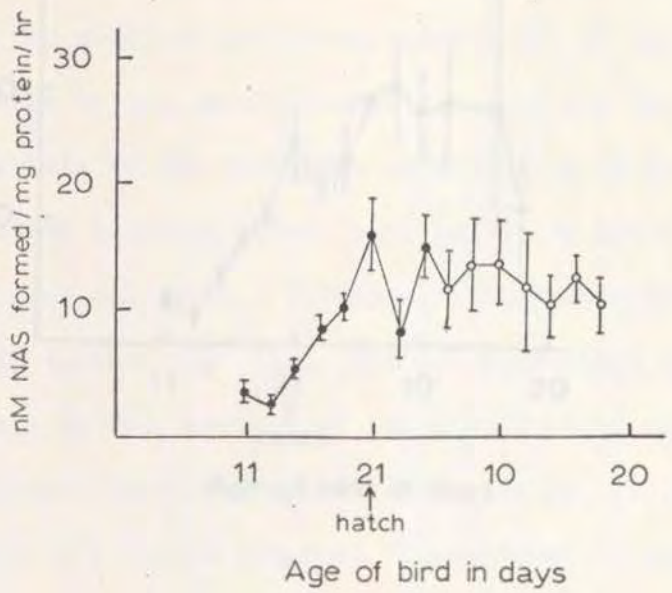


Figure 47: The ontogeny of serotonin N acetyl transferase activity in the pineal gland of chicks raised in diurnal lighting. Chicks which were hatched and raised in the dark were moved to diurnal lighting conditions (L:D,14:10) at 3 days of age. Enzyme activity was measured in the pineals taken from birds at the age indicated. Each point is the average of 3 determinations and the bars indicate one standard deviation.

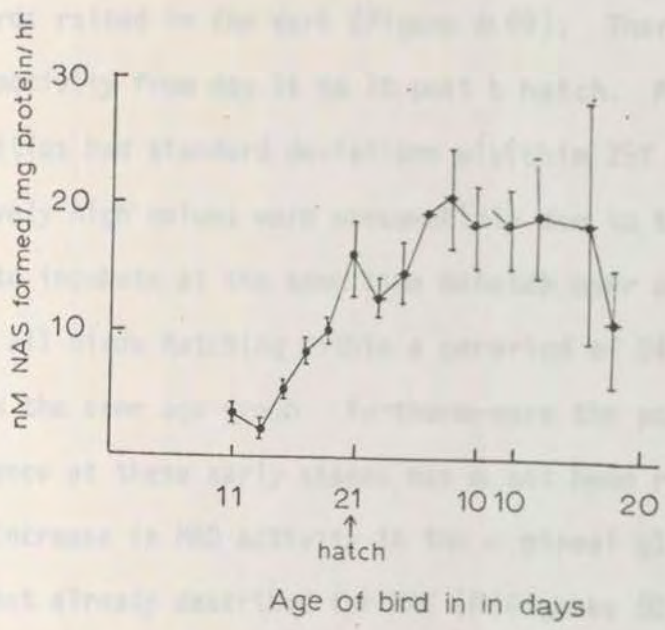


Figure 48: The ontongeny of serotonin in N acetyl transferase activity of the pineal glaland of chicks raised in continuous lighting. Cl Chicks were hatched in the dark and transfered to to continuous lighting immediately. Each value i: is the average of at least 3 determinations and nd the bars indicate one standard deviation.

those for birds raised in the dark (Figure 49). There was a decrease in activity from day 16 to 18 post hatch. Most of the enzyme activities had standard deviations within 25% of the mean. These relatively high values were presumably due to the fact that eggs put in to incubate at the same time hatch over a period of 48 hours and all birds hatching within a period of 24 hours were classified in the same age group. Furthermore the possibility of a sex difference at these early stages has not been ruled out.

The increase in MAO activity in the pineal gland was quite similar to that already described for NAT (Figures 50, 51, 52, 53). Birds raised in the dark showed greatest fluctuations in enzyme activity. Moreover adult levels of activity were exceeded between day 2 to 16 post hatch. There was a 6 fold increase in specific activity between the 11 day embryo and the adult and a 43 fold increase in total activity per pineal gland.

4. Development of NAT and MAO Activities in the Cerebellum

As there were no significant differences in enzyme activity between birds raised under either diurnal lighting or continuous darkness, the data were pooled. Furthermore no essential differences in the shape of the ontogeny curves were observed whether the enzyme activity was expressed on a mg wet weight, mg protein, or mg DNA basis. 70% of adult NAT activity was observed as early as the 11 day embryo cerebellum (Figure 54) and by day 10 post hatch adult activities were

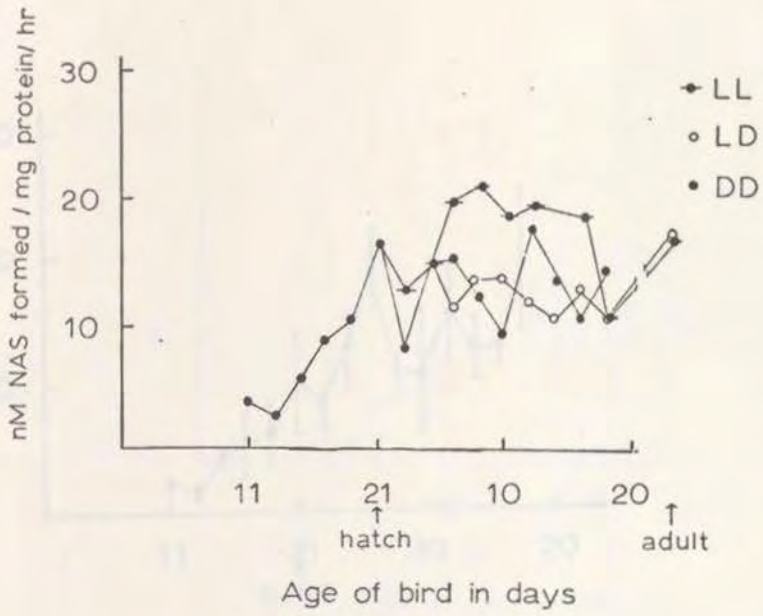


Figure 49: A comparison of the ontogeny of serotonin N acetyl transferase activity in the pineal glands of chicks raised in several conditions of lighting. This is a composite drawing of the data shown in figures 46, 47, 48.

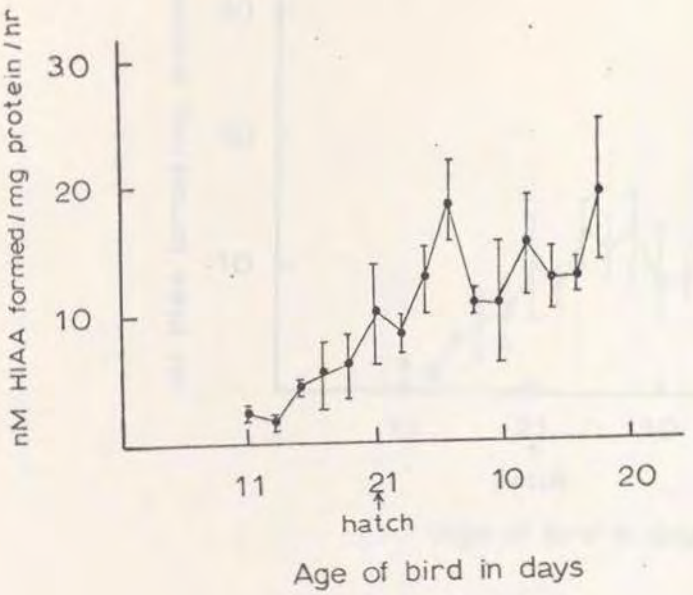


Figure 50: The ontogeny of monoamine oxidase of chicks raised in the dark. Pineals were taken from chicks or chick embryos at the age indicated and enzyme activity was measured as described in methods. The bars indicate the standard deviation and each point is the average of 3 or more determinations.

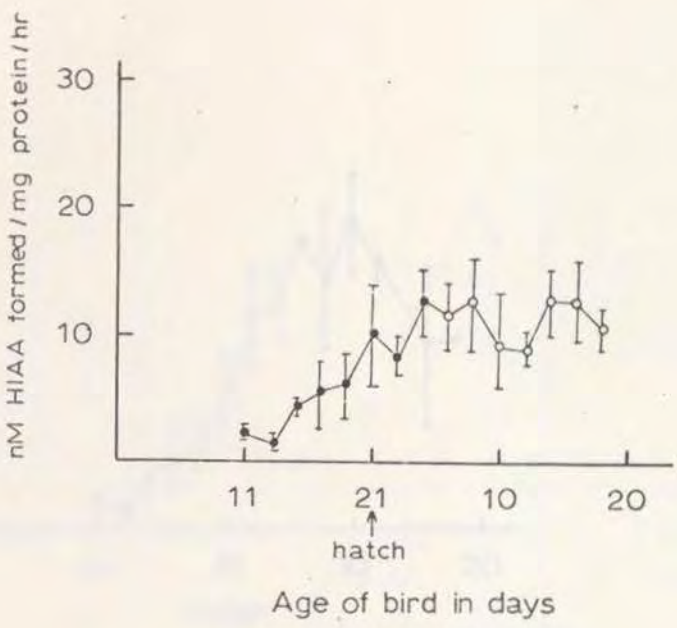


Figure 51: The ontogeny of monoamine oxidase activity in the pineal gland of chicks raised in diurnal lighting. Chicks which were hatched and raised in the dark were moved to diurnal lighting conditions (L:D, 14:10) at 3 days of age. Enzyme activity was measured in the pineals taken from birds at the age indicated. Each point is the average of 3 determinations and the bars indicate one standard deviation.

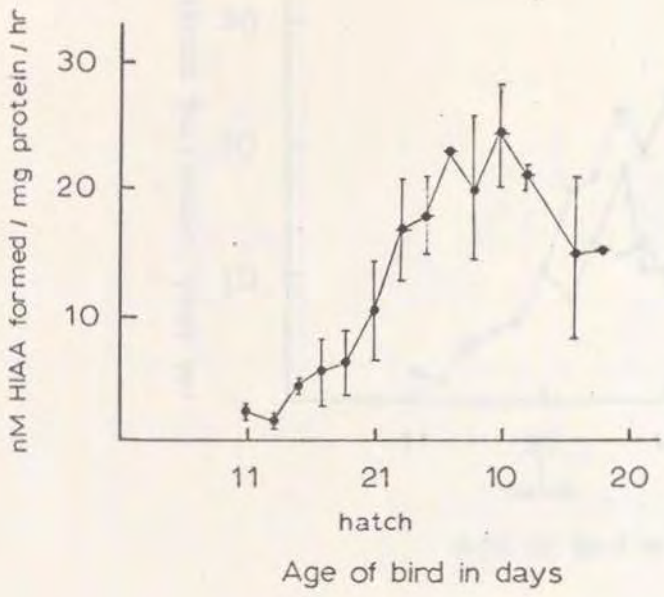


Figure 52: The ontogeny of monoamine oxidase activity of the pineal gland of chicks raised in continuous lighting. Chicks were hatched in the dark and transferred to continuous lighting immediately. Each value is the average of at least 3 determinations and the bars indicate one standard deviation.

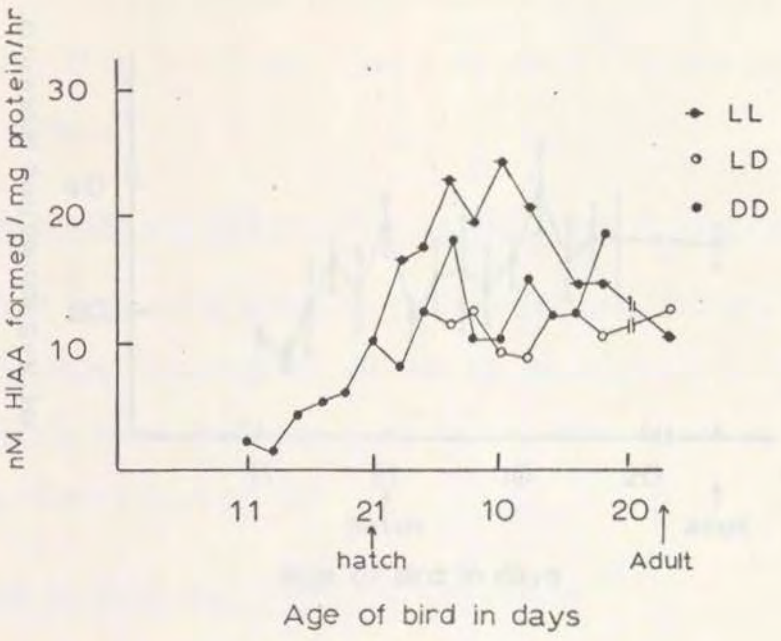


Figure 53: A comparison of the ontogeny of monoamine oxidase activity in the pineal glands of chicks raised in several conditions of lighting. This is a composite drawing of the data shown in figures 50, 51, 52.

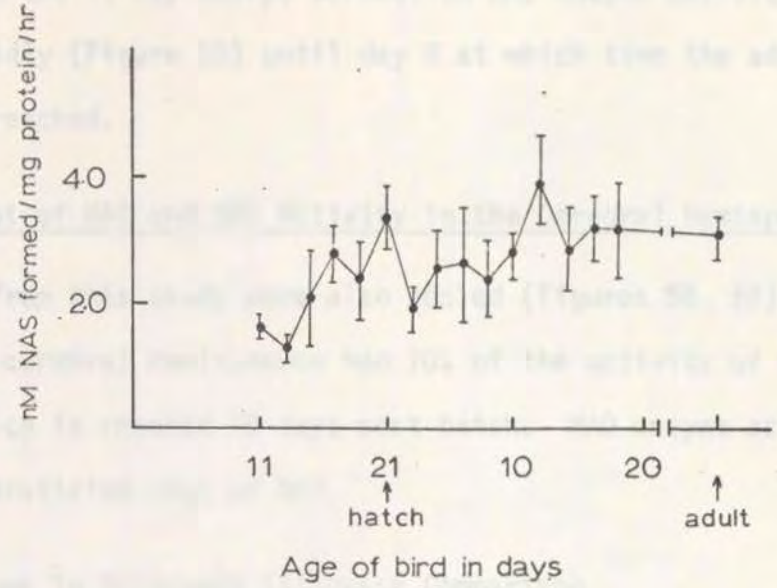


Figure 54: The ontogeny of chick cerebellar serotonin N acetyl transferase activity. As there were no significant differences in enzyme activity for birds raised in the dark or under diurnal lighting conditions data were pooled. Each value is the mean of 6 or more determinations and the bars indicate one standard deviation.

reached. From the 11 day embryo cerebellum MAO enzyme activity increased rapidly (Figure 55) until day 8 at which time the adult activity was reached.

5. Development of NAT and MAO Activity in the Cerebral Hemispheres

Data from this study were also pooled (Figures 56, 57). The 11 day embryo cerebral hemispheres had 70% of the activity of the adult bird which is reached 18 days post hatch. MAO enzyme activity development paralleled that of NAT.

6. Adult Values in Different Tissues:a Comparison

NAT activity in the pineal gland was approximately 6 fold that found in the cerebellum and 3 fold that found in the cerebral hemispheres (Table 12). MAO activity in the pineal gland was double that in either the cerebellum or cerebral hemispheres. On a DNA basis the cerebral hemisphere had 6 times the NAT activity of the cerebellum whereas on a mg wet weight basis or mg protein basis there was only a two fold difference in activity. MAO activity (nM/mg DNA/hr) was 2-2.5 fold greater in the cerebral hemispheres than in the cerebellum whereas the cerebellum showed slightly higher activity when expressed on either a mg wet weight or mg protein basis.

7. Preliminary Organ Culture Experiments

Pineals were removed from 4 day old chicks and placed in

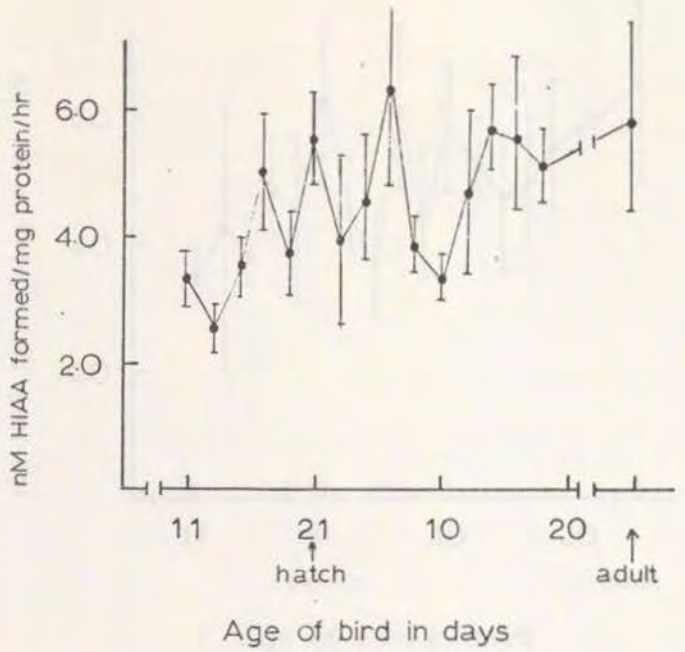


Figure 55: The ontogeny of chick cerebellar monoamine oxidase activity. As there were no significant differences in enzyme activity for birds raised in the dark or under diurnal lighting conditions data were pooled. Each value is the mean of 6 or more determinations and the bars indicate one standard deviation.

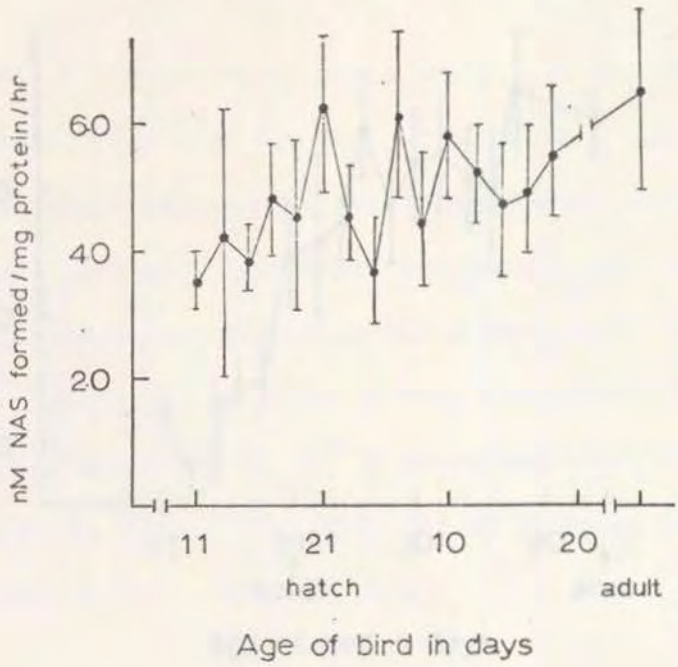


Figure 56: The ontogeny of serotonin N acetyl transferase activity in the chick cerebral hemispheres As there were no significant differences in enzyme activity for birds raised in the dark or under diurnal lighting conditions data were pooled. Each value is the mean of 6 or more determinations and the bars indicate one standard deviation.

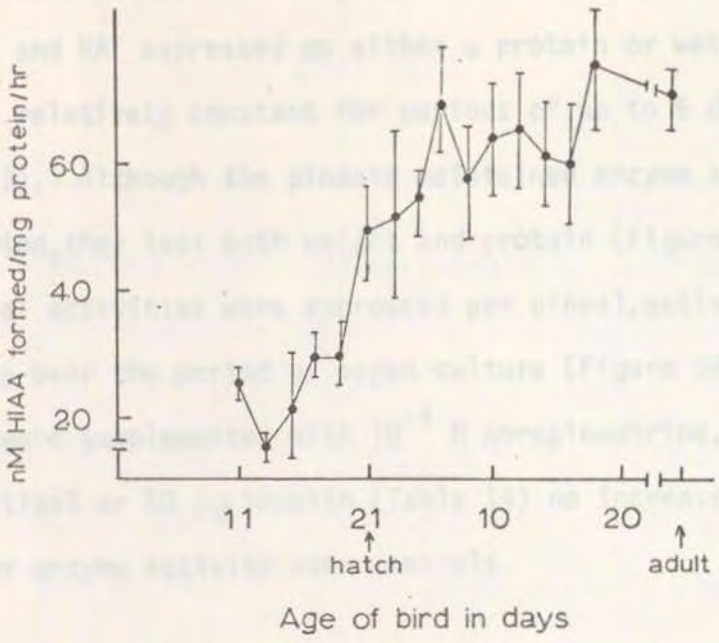


Figure 57: The ontogeny of monoamine oxidase activity on the chick cerebral hemispheres. As there were no significant differences in enzyme activity for birds raised in the dark or under diurnal lighting conditions data were pooled. Each value is the mean of 6 or more determinations and the bars indicate one standard deviation.

organ culture for periods ranging from 2 to 6 days. Activities of both MAO and NAT expressed on either a protein or wet weight basis remained relatively constant for periods of up to 6 days in culture (Table 13). Although the pineals maintained enzyme activities over this period, they lost both weight and protein (Figure 58a). When total activities were expressed per pineal, activities decreased gradually over the period of organ culture (Figure 58b). When the pineals were supplemented with 10^{-4} M norepinephrine, 10^{-5} M hydrocortisol or 50 γ insulin (Table 14) no increase was noticed in either enzyme activity over controls.

V. Discussion

Levels of pineal NAT activity in birds maintained under diurnal conditions of lighting exhibit a diurnal cycle. Maximal levels of activity observed during the dark period were 5-6-fold greater than minimal levels found during the light phase (Figures 42, 43). This finding is in qualitative agreement with recently reported observations of Binkley et al. (1973). However, the extent of the increase in enzyme activity during the dark period found in this study differs substantially from the 27-fold increase reported by the latter authors.

TABLE 13

Days in Culture	nM/mg wet wt/hr	nM/mg protein/hr	nM pineal/hr	mg wet weight
		NAT		
2	1.04 ± .228	10.9 ± 1.3	1.03 ± .31	.97 ± .13
4	1.13 ± .36	12.5 ± 5.4	.89 ± .17	.82 ± .16
6	1.06 ± .23	13.2 ± 2.4	.53 ± .01	.61 ± .13
		MAO		
2	1.12 ± .28	11.9 ± 2.6	1.03 ± .21	
4	0.99 ± .35	11.5 ± 3.8	.79 ± .08	
6	0.87 ± .27	12.6 ± 2.1	.54 ± .28	

Activities of NAT and MAO of pineals from 4 day old birds raised in the dark and placed for up to 6 days in organ culture. Values are the average of three determinations ± standard deviation.

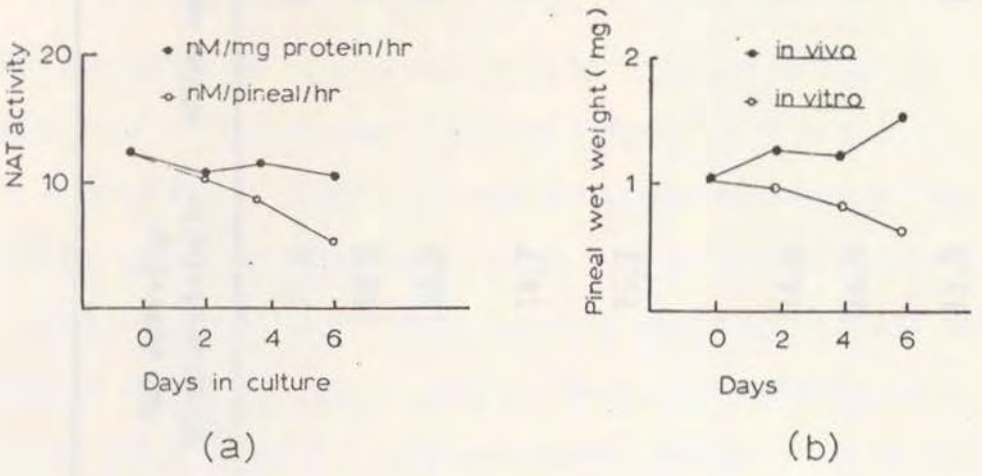


Figure 58: The activity of serotonin N acetyl transferase in pineals maintained in organ culture for periods of up to 6 days. NAT activity was measured as described in methods. The in vivo ontogeny of NAT is added as a comparison with the changes of pineal NAT activities after the corresponding period in organ culture.

TABLE 14

Condition	NAT Activity		MAO Activity	
2 → 4 day	nM/mg protein/hr	nM/mg wet weight/hr	nM/mg protein/hr	nM/mg wet weight/hr
in vivo	12.7	I.32	11.6	1.33
in vitro	13.1	1.30	12.9	1.30
+ 10^{-4}M norepinephrine	14.7	1.46	13.2	1.39
+ 10^{-4}M norepinephrine + 10^{-5}M hydrocortisol	14.1	1.45	14.7	1.53
10^{-4}M norepinephrine + 10^{-5}M hydrocortisol + 50 γg insulin	16.0	1.62	15.7	1.62
2 → 3 day				
in vitro	15.5	1.26	14.8	1.26
+ 10^{-4}M norepinephrine	14.3	1.27	14.8	1.26
+ 10^{-5}M hydrocortisol + 50 γg insulin	14.9	1.22	13.3	1.22

Pineal organs in culture: the effects of various additives. See methods for further detail.

The reasons for this discrepancy in the apparent extent of cyclical variation in levels of enzyme activity are not known. Although both studies were made with White Leghorn chickens, it seems highly probable that different lines of bird were used and the possibility of genetic differences affecting levels of NAT activity cannot be eliminated. Birds used in the two studies were of different ages, and it seems possible that the extent of the range of daily variation in enzymic levels has not attained its maximal value by the 18th day post-hatch, the age of birds used in this study.

However, the most probable source of the quantitative discrepancy between my observations and the data of Binkley et al. (1973) would appear to be the difference in procedures used to assay NAT enzyme activity. The latter workers used the assay method of Klein et al. (1969). This method is based upon the resolution by two-dimensional TLC of pineal metabolites of tryptophan, which appear to include melatonin. However, the modified assay procedures developed for use in this study clearly demonstrate that the substance presumed to be melatonin contains an as-yet-unidentified metabolite of tryptophan (Figure 37). This material is probably a product of serotonin metabolism and may be 5-OH-formyl-Kynurenamine or 5-OH-Kynurenamine, recently reported to be formed by extracts of rabbit brain (Hirata et al., 1974). It therefore seems probable that my assay procedure gives more accurate values of NAT activity than

those obtained with the procedure of Klein et al.

The diurnal cycle in pineal NAT level observed here (Figure 42) and by others (Binkley et al., 1973) affords the possibility of resolving the controversy regarding direct light sensitivity of the avian pineal (Ralph, 1970). Nagel et al. (1972) have obtained conclusive evidence of a direct response of explanted duck pineal to lighting conditions in organ culture. However, no definitive study has yet been reported for the pineal of the domestic chicken, gallus gallus. My results (Figures 42, 43) warrant prediction of the following possible results to be obtained in experiments with blinded birds. If the rhythm remained normal then it would be clear the pineal could receive information about lighting either directly or indirectly from other areas of the brain which are light sensitive. It may be recalled that a nervous connection has been reported between the third ventricle and the pineal stalk in the pigeon (Ueck, 1970). On the other hand if the cycle was abolished under all lighting conditions in these blinded birds (e.g., erratic NAT activities similar to those of normal birds maintained in the dark) then it would be an indication that the pineal receives lighting information solely by way of the eyes.

A circadian rhythm of NAT activity has been established for the rat (Klein et al., 1970) and occurs as early as 8 to 10 days after birth (Ellison et al., 1972). There is suggestive evidence

that such a rhythm occurs in the chick as early as 2 days post hatch (Lynch, 1971). This rhythm has been demonstrated in both 18 day (Figure 42) and 8 week old chicks (Binkley et al., 1973). Therefore the ontogeny of both NAT and MAO was studied in birds raised under the three previously described lighting conditions. Their developmental pattern is similar to that of neural development as monitored by the increase in acetylcholinesterase activity (Burdick and Strittmatter, 1965) or the extend of myelination (Garrigan and Charaff, 1963). The greatest rate of increase in NAT enzyme activity which occurs between day 13 and 19 coincides, if only fortuitously, with the enhanced secretory activity of the embryonic pineal (Figure 49) described by Campbell and Gibson, 1970. Although HIMOT activity is low at this stage (Wainwright, 1974b) as compared to adult value (10 nM melatonin/mg protein/hour v.s. 150 nM melatonin/mg protein/hour), it is still present in measurable amounts.

Klein et al. (1970) have suggested that NAT may be a key enzyme in the control of melatonin synthesis. Since NAT activity shows a pronounced increase prior to hatch there is a distinct possibility that melatonin may be one of the products secreted by the pineal at this early stage of development and also act as one of the factors involved in the control of brain development.

There are substantial similiarities between the course of development of both NAT and MAO activities in the chick and the rat pineals. In chick the most rapid increase in enzyme activity occurs

prior to hatch (Figures 49, 53) and birth (Ellison et al., 1972; Snyder, 1968) respectively.

Chicks raised under continual lighting are more active, develop faster and eat more than controls raised under diurnal lighting (McCuaig, 1974). The greater increases in both NAT and MAO activities under constant lighting (Figures 49, 53) may be another result of this general stress.

The NAT activity of 3 week old birds (1:6 $\pm$ 0.48 nM/pineal/hr) is slightly less than the value of 1.8 nM/pineal/hr reported by Binkley et al. (1973) for 8 week old birds. NAT activity at hatch is 10 times the activity reported for rats at birth (Ellison et al., 1972) and is consistent with the greater activities in the chick already reported (Binkley et al., 1973). MAO activity in the adult hen is approximately two fold higher than that reported by Axelrod et al., (1964). They used /^{14}C/ tryptamine as substrate for the enzyme rather than serotonin and the methods of assaying for MAO were completely different.

Although the chick pineals in organ culture lost both weight and protein we did not notice the 50% decrease in NAT activity that has been reported in rat pineals placed in organ culture for a 24 hour period (Klein and Berg, 1970). Nevertheless, after 6 days in organ culture there was a progressive loss in NAT activity and the development of the pineal in culture was different

from that already described for the pineal in vivo. In preliminary experiments the addition of norepinephrine at concentrations used by Klein et al. (1970) did not cause a substantial increase in NAT activity and it is possible that norepinephrine is not controlling NAT at this stage in development at the concentration studied. There is much evidence that norepinephrine is a factor in the control of NAT activity in adult rat pineals in organ culture (Klein et al., 1970). Whether the chick is less sensitive to norepinephrine at these early stages, or whether it is sensitive at all is not known. A study of the effects of norepinephrine on adult chick pineals should be done in order to make a better comparison.

The ontogeny of MAO and NAT in both the cerebellum and cerebral hemisphere parallels the development of neural activity in the brain as measured by electrical activity (Corner et al., 1967) and synapse formation (Burdick and Strittmatter, 1965). In whole embryonic chick brain MAO activity increases 30% between days 11 and 20 prehatch (Bourne, 1965). Eiduson (1966) reported that MAO was 50% higher in the cerebral hemispheres as compared with the cerebellum. Our results are consistent with these findings.

VI. SUMMARY AND CONCLUSION

1. The ontogeny of both NAT and MAO was examined in the pineal gland from the 13 day embryo until the 18 day hatched bird for birds raised under three different lighting conditions; L:L, L:D, D:D. A comparison of the data showed that birds raised under continuous lighting had significantly higher activities of both enzymes at 8 and 10 days of age. Nevertheless, values were similar at 18 days of age and in the adult pineal. Whether this temporary increase is indicative of the conditions of stress was not determined.

2. The ontogeny of both enzymes in the pineal, cerebellum and cerebral hemispheres parallel the development of brain neurological activity. There is a distinct possibility that the material secreted from the pineal into the cerebral spinal fluid prior to hatch may contain melatonin. The activity of NAT approached adult levels just prior to hatch.

3. Pineal NAT has a circadian rhythm similar to the one already described for the rat. It showed a 6 fold increase in activity at night after the lights were off provided the animal was kept under diurnal lighting conditions. Continuous lighting abolished the rhythm and erratic values resulted when birds were kept in constant darkness. When the lights were turned on in the dark portion of a diurnal light cycle activity fell to daytime levels after 15 minutes. Neither the cerebellum or cerebral hemisphere NAT activities showed this rhythm. MAO from the 3 tissues

previously described showed no evidence of having a rhythm either.

4. Pineals grown in organ culture slowly lost enzyme activity over the period of incubation and did not mimic the in vivo growth under the conditions we have used.

5. The addition of norepinephrine to 4 day old chick pineals in organ culture did not cause a significant increase in NAT activity as has been reported for the rat pineal even though similar concentrations of norepinephrine were used. Whether the chick pineal is less sensitive to norepinephrine or whether it is sensitive at all is not known.

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Canadian Federation Proceedings 12: 276.

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The isolation of pig haptoglobin and the separation of its complex with hemoglobin into polypeptide chains.

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