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OBSERVATIONS ON THE NATURE AND THE INTERACTION OF A CAROTENOPROTEIN AND A CYTOCHROME FROM THE EGGS OF A PROSOBRANCH MOLLUSC

a Thesis presented by

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ABSTRACT

Evidence is given for interaction of the carotenoglycoprotein ovorubin and a haemoprotein, spectroscopically indistinguishable from the cytochrome-like helicorubin of *Helix pomatia*, found in the egg jelly of the prosobranch *Pomacea canaliculata*.

Ovorubin catalyses reduction of helicorubin by NADH, NADPH, GSH and ascorbate. Addition of ATP, ADP and AMP to the egg homogenate elicits changes in the Fe^{3+}/Fe^{2+} ratio of the haemoprotein. These are dependent on the initial ratio and the nucleotide concentration. ATP synthesis could sometimes be shown in homogenates of eggs developed for 100 hrs and correlated with redox changes in helicorubin. No ATP synthesis is observed with ovorubin-free homogenates or those of freshly laid eggs. Returning ovorubin to homogenates freed from this protein does not restore ATP synthesis. Egg homogenates dephosphorylate ATP and ADP. Some 10% of this activity remains in chromatographically prepared ovorubin. Extensive purification results in loss of this activity.

Ovorubin, but not its apoprotein, undergoes spectral changes, reversed by light, on exposure to carbon monoxide.

E.s.r. spectra of ovorubin show signals at $g = 1.96$ and 4.2, suggestive of non-haem iron.

Electron micrographs of ovorubin, together with a sub-unit structure determined by SDS-PAGE, are used to suggest a molecular model.

Helicorubin from *P. canaliculata* has been purified and found to be a basic protein.

Ovorubin, added to oxidised helicorubin or cytochrome *c* from horse heart or *Candida*, gives partial reduction in dark. In visible light, reduction is virtually complete. With apoprotein partial reduction occurs in light or dark to the degree found with the carotenoprotein in the dark.

Reduction of cytochrome by ovorubin or its apoprotein is competitively inhibited by superoxide dismutase indicating generation of $O_2^{\cdot-}$.

Anaerobically in the dark, reduction of cytochrome by ovorubin and apoprotein reaches about one third the level with ovorubin aerobically in the light and one half that with ovorubin aerobically in the dark. These observations suggest the presence of three redox centres on ovorubin. The functional significance of these interactions, especially during embryonic development, is discussed.

SYMBOLS AND ABBREVIATIONS

α,β-CH_2ADP	α,β-methyleneadenosine 5'-diphosphate
Ap_5A	p^1p^5-di adenosine pentaphosphate
BPB	bromophenol blue
CaP	calcium phosphate gel
Con A-Sepharose	Concanavalin A-Sepharose
DEAE	diethylaminoethyl cellulose
GSH	reduced glutathione
HR	helicorubin
K_R	retardation coefficient
M	electrophoretic mobility
M_o	free electrophoretic mobility
MW	molecular weight
OR	ovorubin
PAS	periodic acid Schiff's stain
PEI	polyethylimine
PAGE	polyacrylamide gel electrophoresis
R_F	relative electrophoretic mobility
R_p	phosphorylation state ratio = $[ATP]/[ADP][P_i]$
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
St.Dev.	standard deviation
T	g acrylamide plus g bisacrylamide per 100 ml solution
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine
I I	The bars represent the scatter, • and Δ the means of determinations.

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CHAPTER 1.

GENERAL INTRODUCTION

1.1. The eggs of *Pomacea canaliculata australis*

The eggs of most species of prosobranch gastropods of the genus *Pomacea* contain red, orange or green pigments (Vilella, 1956). The species with which we have been most concerned, *Pomacea canaliculata australis* (d'Orbigny) lays rose-red egg masses on solid objects outside the water. The eggs are some 3 mm in diameter. The ovum, according to Hylton-Scott (1934), is about 50 µm in diameter and surrounded by the egg jelly within a calcareous shell. The ovum thus accounts for roughly one part in 2×10^5 of the total egg mass. Depending on environmental conditions (temperature, light), the ovum reaches the 16-blastomere stage within 12-20 hours. Unfertilised eggs usually dry out gradually. The egg jelly of molluscs has been commonly regarded as an inert and undifferentiated fluid, serving as a nutrient medium for the embryo.

Comfort (1947, 1949) showed that the pigmentation of the eggs is predominantly due to a heat-stable carotenoid-protein complex, which is susceptible to alcohol, acetone and pyridine dissociation.

Further studies by Cheesman (1954, 1956, 1958) showed that when a homogenate of these eggs is made with water in the Waring blender and centrifuged for 15 minutes at 10,000g, a turbid supernatant is obtained in which all the red pigment is dissolved. The absorption spectrum of this solution shows maxima at 427, 510, 545 nm, and an inflexion at 480 nm.

1.2. Survey of earlier literature on the major protein components present in the eggs.

1.2.1. Helicorubin

The maximum at 427 nm is attributed to that of the Soret band of a haemochromogen spectrophotometrically indistinguishable from helicorubin. Helicorubin was first described by Sorby (1876), who found that the crop and intestine of the garden snail contain a red-brown fluid which has an absorption spectrum resembling that of a haemochromogen also found in

the digestive gland and other organs. On exposure to air, the pigment underwent oxidation with concomitant disappearance of its strong absorption bands. These bands reappeared however, when the fluid was made alkaline and the pigment became reduced. These observations were confirmed by Krukenberg (1882) who named the pigment helicorubin, and by MacMunn (1883, 1885, 1886a,b) who named it enterohaematin.

Purification was first attempted by Dhéré and Vegezzi (1917a,b) and Dhéré (1919) who ultrafiltered snail "bile" and separated a red solution containing helicorubin (the name now accepted) from a brown pigment. This alkaline helicorubin has a haemochromogen type of absorption spectrum consisting of a sharp α-band at 562.5 nm, a weaker β-band at 529.5 nm, and a Soret band at 427.5 nm. On acidification these sharp bands are replaced by two diffuse bands in the red, with the β-band now more pronounced, and the Soret band displaced to 415 nm. Dhéré and Vegezzi (1917b) concluded that helicorubin and haemoglobin have the same prosthetic group. The claim by Anson and Mirsky (1925) that helicorubin is capable of undergoing oxygenation as well as oxidation, has not been confirmed.

Studies by Roche and Morena (1936a,b) on the variation of the degree of reduction of partially purified helicorubin with pH, showed that 50% reduction occurred at about pH 7.3. They suggested that the reduction under alkali conditions may be due either to a reducing substance coexisting with the pigment in the partially purified preparation, or to an active reducing group in the protein moiety of helicorubin.

Although helicorubin was the first haemoprotein of the haemochromogen type to be described, final purification was not achieved until the late 1950's when Keilin (1956, 1957) described the isolation and purification of helicorubin from the gastrointestinal fluid of *Helix pomatia*, where it occurs as an extracellular haemochromogen. She demonstrated that purified helicorubin has many properties in common with bacterial and mammalian cytochromes of both the *b* and *c* type. Preliminary

results (Keilin, 1956, 1957) suggested a molecular weight of 18,500, while later observations (Keilin, 1968) showed that the heat-stable protein has a molecular weight of about 12,000, and an isoelectric point in the region of pH 4.3. It is auto-oxidisable below pH 6.0 and does not react with CO in neutral or alkaline solutions, in contrast to mammalian cytochrome *c*, which reacts with CO below pH 4.0 and above pH 12.0. Table 1.1 compares the main features of the absorption spectra for helicorubin and cytochrome *c* from horse heart.

TABLE 1.1

	α-band	β-band	γ-band (Soret band)	$E_{276}/E_{\gamma ox}$
Preparation	nm	nm	nm	
Helicorubin red.	563	531	428	
ox.			416	0.267
Cytochrome *c* red.	550	520	416	
ox.			410	0.219

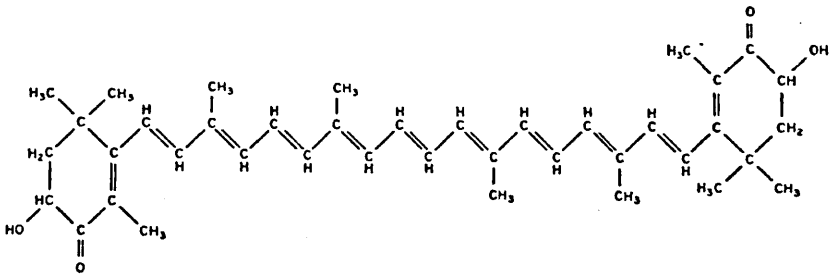
Keilin (1969) used the $E_{276/416}$ and the position of the Soret band as criteria for the purity of the protein. Helicorubin also differs in amino acid composition from other cytochromes in so far as it has a low lysine and a high proline content (Keilin 1968) and methionine is absent. Its overall amino acid composition is closer to that of bacterial than mammalian cytochrome *c*. This is supported by the observation (Keilin, 1968) that helicorubin reacts readily with a particulate oxidase preparation from the bacterium *Azotobacter vinelandii*, but not with mammalian cytochrome *c* oxidase under the same conditions. It suggests that, from an evolutionary point of view, the extracellular haemochromogen from *Helix pomatia* is more closely related to cytochromes of prokaryotes than to those of eukaryotes, an observation which is further supported by immunological studies (Keilin and Orleans, 1969).

1.2.2. Ovorubin

The remaining absorption maxima of the egg homogenate were found to be due to the presence of the carotenoglycoprotein ovorubin. Cheesman (1954, 1956, 1958) isolated ovorubin from the homogenate by adsorption on aluminium hydroxide gel followed by its elution with 0.2 M-Na_2HPO_4. Three adsorptions and elutions normally sufficed to produce a preparation whose absorption spectrum was not altered in the visible and U.V. ranges on further treatment. From this, a provisional criterion of purity was proposed, suggesting that the ratio of ovorubin in 0.2 M-Na_2HPO_4 or water measured against the solvent shall lie in the range of 2.6-2.8. Fresh eggs have to be used to achieve reproducible results. Cheesman (1958) found that ovorubin has a nitrogen content of 12.6 $\pm$ 0.2%N and gives a strong Molisch reaction, indicating that the low nitrogen content may be accounted for by the presence of a large carbohydrate component, which on analysis shows the presence of a 2-amino-2-deoxyglucose, mannose, galactose, and fucose (Norden, 1962), accounting for about 20% of the molecular weight.

In 1976 Uhlenbruck *et al.* showed that ovorubin interacts with the plant lectin concanavalin A, indicating the presence of glucose and/or mannose at the non-reducing end of the carbohydrate moiety.

Cheesman (1968) identified the carotenoid component as astaxanthin:



He attributed the spectral features at 280 nm mainly to the aromatic amino acid residues in the protein component, whereas the inflexions at 330 and 480 nm and the maxima at 510 and 545 nm were thought to be contributed by the carotenoid.

Thoroughly dialysed solutions of ovorubin were found to have a pH of 5.5, suggesting an isoionic point in this region. Norden (1962) showed that the carotenoprotein is homogeneous by electrophoresis in 0.12 M veronal buffer (pH 8.9) and moves towards the anode. Her work showed that ovorubin is a strong proteinase inhibitor which inhibits the action of trypsin, chymotrypsin, and possibly that of takadiastase proteinase and subtilisin (Norden 1962, 1972). She suggested that ovorubin, which is quantitatively the most important nitrogen component of the egg, and which appears to be partially utilised during the development of the snail embryo (Cheesman 1958), combines the properties of at least two proteins found in the eggs of the domestic fowl, i.e. those of a storage protein and a polyvalent proteinase inhibitor. Ovorubin was found to be remarkably heat-stable (Cheesman, 1958). Heating a solution of pH 4-7 at 100 ^{o}C for long periods produces but a slight turbidity, associated with a reversible partial dissociation of the carotenoid group from the protein. It has a low surface activity, with little tendency to froth or form unimolecular films, suggesting a considerable stability of the native protein. This stability appears to be conferred by the carotenoid, since the apoprotein, which is readily isolated by treatment of the chromoprotein at 0 ^{o}C with ice-cold acetone, has much greater surface activity. Apo-ovorubin is readily soluble in water and rapidly denatures when heated in solution above 70 ^{o}C. Since the apoprotein still contains the carbohydrate moiety, the heat-stability of the intact chromoprotein must be a function of the carotenoid group and this is supported by the finding that on recombination of the apoprotein and carotenoid in an acetone-water mixture, the heat stability of the protein is restored.

In view of the heat-resistant properties of ovorubin, Cheesman (1958) has suggested that the function of ovorubin is

a) to assist water conservation by the eggs at high temperatures, since native proteins have a much higher water binding capacity, and

b) to protect the embryo from harmful radiation, since the snail is a tropical species and the eggs are laid in places exposed to intense sunlight.

It is interesting to note in this context that the absorption spectrum of ovorubin approximately corresponds to the solar energy spectrum with a peak around 500 nm.

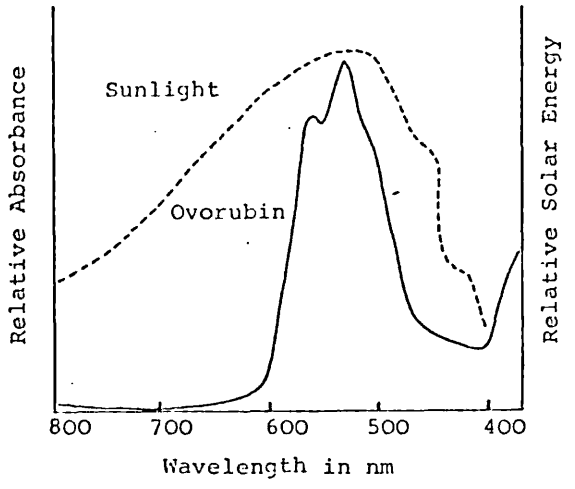


Fig. 1.1. Absorption spectrum of ovorubin compared to the solar spectrum that strikes the earth.

After Wolken, 1975.

1.3. Possible transfer of maternal proteins to an embryo.

The red colour due to ovorubin appears to be removed progressively from the egg jelly to the embryo during development. The newly hatched snails have transparent shells, through which can be seen the red digestive gland, of the same colour as the eggs in the early stage of development. As already mentioned, this pigmentation of the egg and digestive gland is due mainly to ovorubin and helicorubin which can represent

up to 60% of the total protein content of freshly laid eggs. At the time of hatching at least 60% of the ovorubin originally present in the jelly has been incorporated in the hepatopancreas of the animal (Cheesman and Helm, 1977) After about a week, the shells turn dark, and the pigmentation of the digestive gland becomes quantitatively less important. During the same period, the animals, originally showing positive phototaxis, lose this characteristic. If it can be excluded that these two proteins are broken down and resynthesised in the developing embryo, it would appear that proteins of maternal origin are transferred to the young snail (Cheesman and Helm, 1977).

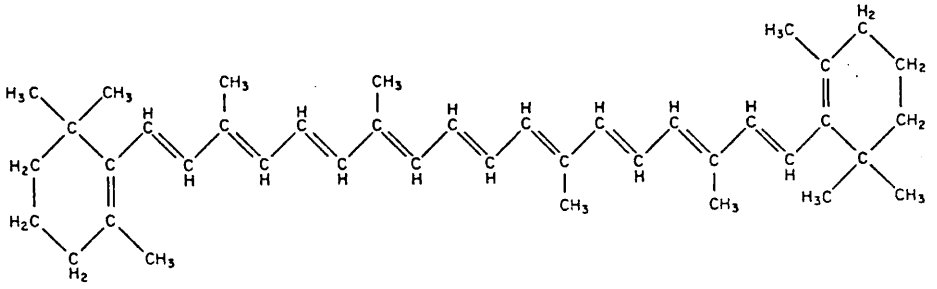
Somewhat similar observations have been reported by Benjamin and Walker (1971), who worked with the pond snail *Lymnaea stagnalis*. The nervous system of these snails displays a strong orange-red pigmentation. The intraneuronal pigment renders these nerve cells sensitive to directly projected white light, and changes in membrane potential can be observed on illumination. Purification of these pigments from "brain" homogenates led to the isolation of a red and a yellow chromoprotein. They identified the "red fraction" as a haemo-protein and suggested that it closely resembled Keilin's (1957) helicorubin, although there can be little doubt that it is myoglobin The "yellow fraction" showed a protein with two main absorption peaks in the 456-458 nm and 484-486 nm region. Treatment of this protein with organic solvents and subsequent thin-layer chromato-graphy produced seven fractions closely similar in absorption characteristics. It was suggested that the major carotenoid component was lutein.

They also showed that newly hatched snails contained these orange and yellow pigment granules in the neurones of their central nervous system and proposed that the pigment must have originated from the eggs since carotenoid synthesis had never been observed in animal tissue.

1.4 Carotenoids and retinenes

1.4.1 Structure and definition of carotenoids

Carotenoids are a widely distributed group of pigments. They are present in all higher plants and many protista, both photosynthetic and non-photosynthetic, and are also found in many animals. Essentially, they are tetraterpenes, consisting of eight isoprene units which arise through the tail-to-tail condensation of two identical 20 C units. This series of conjugated olefinic bonds constitutes the chromophoric group of a carotenoid. The energy required for $\pi \rightarrow \pi^*$ transition is reduced by the extensive conjugation resulting in an absorption in the visible range. Most naturally occurring carotenoids are yellow, orange or red, although there are colourless ones. Carotenoids are usually cyclised, although cyclisation is not as extensive as in triterpenes (e.g. sterols). This inclusion in a ring forces the diene to adopt a cisoid conformation, which again leads to an increase in λ_{max}. Typical of "cyclic carotenoids" is β-carotene which contains 2 β-ionone residues in its molecule.



β-Carotene structure.

Carotenoids are subdivided into -

i) C_{40} hydrocarbons

ii) C_{40} xanthophylls, which can be further subdivided into mono-, di-, and poly-hydroxy compounds, ethers, aldehydes, ketones and acids

iii) carotenoids with more than 40 C atoms in their carbon framework

iv) apocarotenoids with less than 40 C in the carbon framework.

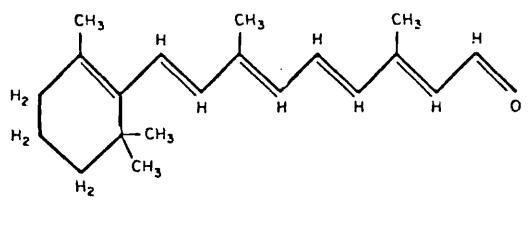
1.4.2. Retinenes

While carotenoids are present in plants and many protista, animals cannot synthesise them and most include them in their nutrition for the formation of a class of essential growth factors belonging to the vitamin A group. These latter have been called retinenes because they invariably form part of the visual pigments in both vertebrate and invertebrate eyes.

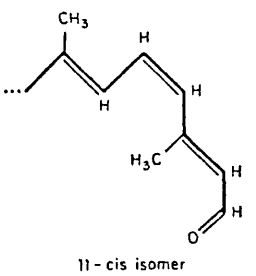
Retinenes are twenty-carbon polyprenyl alcohols formed by the central cleavage of forty-carbon carotenoids.

In 1944 Morton established the identity of retinene with vitamin A aldehyde and demonstrated its importance as an intermediate in vitamin A synthesis. Vitamin A is essential for normal growth and development of animal tissue (Moore, 1957).

In halophilic bacteria, retinal (vitamin A aldehyde) is the essential chromophore of a protein acting as a light-operated hydrogen ion pump coupled to ATP-synthesis.



all-trans-Retinene (vitamin A aldehyde)



11-cis isomer

1.4.3 Carotenoid-protein complexes in invertebrates

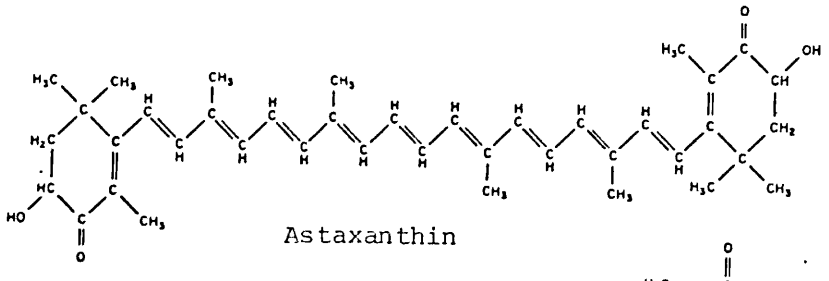
In invertebrates, carotenoid-protein complexes seem to be found predominantly in two general body areas, in the exoskeleton and the epidermis, and in reproductive organs and eggs (Cheesman *et al.* 1967). Cheesman *et al.* (1967) and Zagalsky (1976) differentiate between two quite different types of carotenoid-protein complexes: those in which the carotenoid is associated with a lipoprotein, and those where it is bound stoichiometrically to a simple protein or glycoprotein.

1.4.4 Carotenoid-lipoproteins

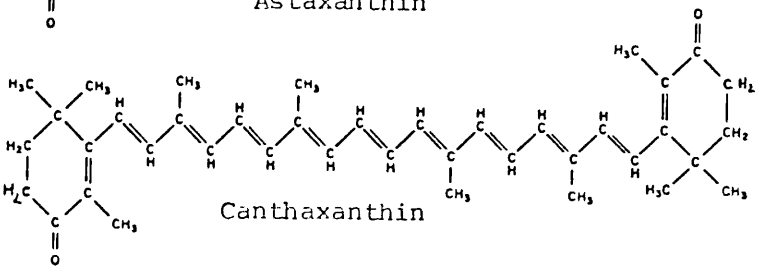
In such complexes, the carotenoid often appears to form part of a lipid prosthetic group, which might act as a "solvent" for the chromophore, and shows no specific interaction with the protein such as would be manifested in a considerable change in the absorption spectrum of the carotenoid.

This type of combination appears to occur in proteins of the eggs and ovary of *Cancer pagurus* (λ_{max} 470,490 nm) (Zagalsky *et al.* 1967) or those of the eggs, ovary, and blood of *Emerita analoga* (λ_{max} 463,472 nm) (Gilchrist and Lee, 1972), where no selectivity of binding is apparent, since all the carotenoids found in the whole tissue are present in about the same relative proportions. However, in an ovary protein of *Pecten maximus* (λ_{max} 472,498 nm) (Zagalsky *et al.*, 1967) polar carotenoid appears to be preferentially combined, and the resultant bathochromic shift has been explained by refractive index changes associated with the dissolution of the carotenoid in the lipid or protein component of the lipoprotein (Buchwald and Jencks, 1968). A single carotenoid prosthetic group is found in a number of carotenoid-lipoproteins e.g. in *Homarus gammarus*, *Lepas* spp. (Cheesman *et al.*,1967), *Plesionika edwardsi* Zagalsky *et al.*,1967) *Acanthephyra* spp. (Herring,1975) and

Pollicepes polymerus (Holter, 1968). This is found to be astaxanthin. Canthaxanthin occurs in *Artemia salina* (Warner *et al.* 1972) and *Branchipus stagnalis* (Zagalsky and Gilchrist, 1976).



Astaxanthin



Canthaxanthin

A "β-carotene-like" carotenoid is also found in many green and blue egg and ovary carotenoid-protein complexes, although traces of other carotenoids are commonly found (Zagalsky, 1975). It has been suggested that the apparent specificity may be due to the predominance of a single carotenoid in the tissue either as a result of metabolic selectivity (Herring, 1975) or preferential selection of a particular carotenoid during transport and deposition. In these cases, the absorption spectrum of the bound carotenoid is often the same as that of the carotenoid in hexane (e.g. *Polymerus* spp. λ_{max} 470 nm, Holter, 1968) or associated with only a small change in the position of λ_{max} (e.g. *Acanthephyra* spp. λ_{max} 486-498, Herring, 1975), although bathochromic shifts and alterations in the shape of the absorption band of the ketocarotenoid, leading to blue complexes, are commonly observed e.g. *Lepas* spp. λ_{max} 600 nm (Cheesman *et al.* 1967),

P. edwardsi λ_{max} 632,670 nm (Zagalsky *et al.* 1967) or *Branchipus stagnalis* λ_{max} 625,675 nm (Zagalsky and Gilchrist, 1976). It appears likely that different modes of binding of carotenoid occur, since mild treatment which removes some of the bound lipid is associated with a disappearance of the absorption band in the 400-500 nm region. It has therefore been suggested (Lee, 1966; Gilchrist, 1968; Lee and Gilchrist, 1972) that lipid-associated carotenoids cause the 400-500 nm absorption, while the maxima in the 370 and 680 nm region arise from canthaxanthin, which is thought to be more firmly bound. In these complexes, the absorption bands have altered spectral characteristics, such as half-band width, when compared with the same carotenoid dissolved in hexane.

Carotenoid-lipoprotein complexes are usually rather unstable (Cheesman *et al.* 1967). Intense light, heat, changes in pH, metal ions, or storage often lead to a change in absorption spectrum. Solutions of the blue lipoprotein complex of *B. stagnalis* turn red unless chloride ions are present (Zagalsky and Gilchrist, 1976) and the brown protein from *Procambarus* spp. changes reversibly from brown to orange when the pH is increased from 7 to 8 (Fyffe and O'Connor, 1974). Although most of the known carotenolipoproteins form relatively unspecific complexes, there appears to be some specificity of association in some of these complexes. Observations on the effects of disruption and reconstitution of the carotenoid-lipoprotein linkage in the chromoproteins from *Homarus gammarus, Lepas* spp., and *Eu(Pagurus) bernhardus* Cheesman *et al.* 1967), and the finding that the lipoprotein from the gonad of *Pecten maximus*, even though a number of different xanthophylls are present, shows a remarkable constancy in total carotenoid content, indicate that the association is more selective and cannot be explained on the basis of passive solution of the carotenoid in the lipid (Zagalsky, 1964).

1.4.5 Carotenoproteins

In general, there is a distinct difference between the above-mentioned carotenoid-lipoprotein complexes and true carotenoproteins, a term which is limited to proteins with associated carotenoid where a strict stoichiometric relationship between carotenoid and protein has been established (Cheesman *et al.*, 1967), although in some instances, when a true carotenoprotein has a lipid prosthetic group, this may contain carotenoids in non-stoichiometric amounts which may be at least equal in quantity to the specific protein-bound carotenoid. These may modify the colour of the whole complex to a marked degree, a feature that has been observed in the green carotenoprotein of the isopod *Idothea granulosa* (Lee, 1966b).

True carotenoproteins are stable combinations where the carotenoid tends to increase the stability of the tertiary or quaternary protein structure, a phenomenon generally observed with other protein-bound polyenes (Heller and Horowitz, 1973; Gammack *et al.* 1971). Since protein and carotenoid are easily dissociated by treatment of an aqueous solution of the protein with solvents known to destabilise hydrophobic interactions, covalent bonds cannot be involved in the combination. It is sometimes fairly easy to reconstitute the native carotenoprotein from freshly prepared apoprotein and carotenoid. The latter has invariably been identified as either astaxanthin, cantaxanthin or one of their derivatives (Zagalsky, 1976).

True carotenoproteins are found in many invertebrate species, and several have been purified. The position of the absorption maxima of the red to blue complexes ranges from 510 nm in the carotenoglycoprotein ovorubin in the eggs of *P. canaliculata* (Cheesman, 1958) to 655 nm in the carapace pigment of *Cambarus clarkii* (Nakagawa *et al.* 1971).

Molecular weights of carotenoproteins vary from 5×10^4 in carapace carotenoprotein of *Aristeus antennatus* to 1×10^6

in the asymmetrical-mandible carotenoprotein of the same species (Zagalsky *et al.*, 1970). At low ionic strength, many of these invertebrate proteins dissociate to the purple β-forms, which commonly have a molecular weight in the region of 4-5 x 10^4 (Zagalsky, 1976). The apoproteins, which are easily prepared by simple acetone precipitation, are often heterogeneous, with molecular weights between 1.8-2.4 x 10^4 e.g. crustacyanin (Cheesman *et al.*, 1967); Jencks and Buten, 1964) or the mantle protein of *Velella* sp. (Zagalsky and Herring, 1977). In many carotenoproteins the quaternary structure is distinctly influenced by the prosthetic groups (Zagalsky *et al.*, 1970); Zagalsky and Herring, 1972, 1977). The apoprotein of the carotenoglycoprotein ovorubin appears to be a notable exception, in that it retains the quaternary structure of the carotenoprotein (Zagalsky, 1976).

Carotenoid-binding capacity seems to range from one (ovorubin and crustacyanin, Cheesman *et al.*, 1967) to two (*Velella* sp. and *Anomalocera patersoni*, Zagalsky and Herring, 1977) or possibly three moles (*Labidocera acutifrons*, Zagalsky and Herring, 1972) per mole of apoprotein.

Carotenoproteins are characterised by low contents of leucine, a high content of acidic amino acids, in particular aspartic acid, and serine, threonine and proline. This is thought to have some relevance to the binding of the carotenoid (Zagalsky, 1976).

1.4.6. Nature of the carotenoid binding site

At present, one can but speculate on the nature of the carotenoid binding sites in carotenoproteins. The size of the carotenoid is in the region of 28 x 5 Å and therefore of the same order as many apoproteins which are ca 40 Å in diameter or less (Zagalsky *et al.*, 1970, Zagalsky and Herring, 1972). One might therefore expect that a large part of the protein is involved in the binding. The sensitivity of binding to organic solvents clearly indicates non-covalent

bonding, and no evidence has been obtained for labile Schiff base formation between the keto-groups of astaxanthin and the amino groups of crustacyanin, or of linkages involving thiol groups (Buchwald and Jencks, 1968a,b). The proposal that astaxanthin may be bound by labile anhydride bonds to the carboxyl group of aspartic acid has not been confirmed experimentally (Zagalsky and Herring, 1972). The relative insensitivity of the spectrum of astaxanthin to solvents, and the low content of hydrophobic amino acids, render it unlikely that the large bathochromic shifts in the absorption bands of these pigments can be accounted for solely on the basis of hydrophobic bonding (Buchwald and Jencks, 1968a,b). The last-cited authors (1968a) proposed a mechanism to account for the spectral shift in which the carotenoid is distorted on binding to the protein in such a way as to strain selectively the double bonds of the polyene chain. Such distortion would raise the energy of the electronic ground state of the polyene and consequently lower the excitation energy. For such a mechanism to be valid, a specific site on the protein must exist in order to confine the strain to the double bonds.

This mode of binding therefore necessitates a close fit between the polypeptide chain and the methyl groups of the polyene and precise and tight alignment of the β-ionone ring. It is thought that the carotenoid is buried in a hydrophobic area of the protein (Kuhn and Kühn, 1967) with the 4- and 4'-keto groups accommodated near the surface of the protein (Buchwald and Jencks, 1968b).

Zagalsky (1976) believes that the high content of small amino acids will facilitate this intimate fit between polyene and polypeptide backbone and he suggests the probability that tertiary structures of carotenoproteins are made up predominantly of random coil and β conformations. This is supported by the low content of leucine, which is thought

to be the main stabiliser in the internal section of α-helices, and the already mentioned high content of "helix breakers" like proline, serine, glycine, and asparagine. UV CD spectra of crustacyanin and *Velella* carotenoprotein confirm this view. The former has an estimated helical content of about 6% (Buchwald and Jencks, 1968b), while the latter appears to have a conformation composed almost entirely of β-structure and β-bends which will favour the formation of antiparallel β-pleated sheets. Since the repeat distance between similarly oriented groups (6.68 Å) in β-pleated sheets is of approximately the same magnitude as the distance between the methyl groups in the polyene chain, Zagalsky (1976) proposes that the immobilisation of these groups occurs via binding to areas of β-pleated sheet in which small apolar residues such as alanine favour strong hydrophobic interaction, while the β-ionone ring, twisted out of the plane of the polyene chain about the 6-7 single bond, may interact hydrophobically through a methyl group in the 1-position with a flexible residue such as isoleucine of the β-pleated sheet. Since the 4-keto groups are of great importance for recombination with apoprotein, he suggests that the β-ionone rings are bound through these groups by hydrogen bonding to imide groups of peptide bonds located on the peripheral chains of the β-pleated sheet. This type of hydrogen bonding between peripheral and adjacent chains of the β-pleated sheet is thought to achieve, via electron withdrawal, an increase in the strength of hydrogen bonding to the carotenoid, which would not be achieved with the single chain. Interaction of the 5- and 5'-methyl groups with an adjacent hydrophobic residue and hydrogen bonding of the 3S and 3'S hydroxy groups of the carotenoid to neighbouring peptide bond carbonyl groups may further help to strengthen the interaction. Under these circumstances, the relative alignment of parts of the β-structure in binding to the carotenoid will control the twisting of the polyene and determine the absorption maxima of the pigment.

The mechanism outlined above seems to hold for several carotenoproteins where detailed examinations have been made. Recent reconstitution experiments by Shone (1978) on the carotenoprotein from the starfish *Asterias rubens* suggest that the mechanism proposed by Jencks and Buchwald (1968a), even if it is valid, may not hold universally. His observations suggest the presence of binding sites on the protein for both hydroxy and keto groups of astaxanthin. The presence of keto groups at the 4- and 4'-positions on carotenoid were found to be essential for sizeable bathochromic shifts in the spectrum to occur, although it is considered uncertain whether these groups are required merely to provide rigid carotenoid-protein binding or whether they play a direct role in the production of the spectral shift. Resonance Raman studies suggest that the protein-carotenoid interactions involved in colour regulation are confined to the polyene chain of the carotenoid. Some tentative evidence against the direct involvement of ketone groups has also been obtained from studies with astaxanthin epoxides. Shone (1978), in view of the considerable variations in polyene chain conformation of carotenoids successfully recombined with proteins, considers it unlikely that a mechanism requiring a highly specific protein binding site, which is considered essential for the twisting mechanism of Buchwald and Jencks (1968a), can be involved in the binding of carotenoids to the *Asterias* protein. He proposed that mechanisms involving polarisation induced by charged groups in the polyene chain should be considered as an alternative. This would reduce the degree of bond alternation in the polyene chain and effect of bathochromic shift. Such a mechanism could possibly be reconciled with the degree of flexibility shown by the *Asterias* protein.

1.5. Functions of carotenoids

In the "Comparative Biochemistry of the Carotenoids", Goodwin (1952), although unable to generalise about the biological

role of carotenoids, concluded that their function in photoreception and reproduction was related to their ability to absorb light.

1.5.1. Function as accessory pigments in energy transfer

Engelmann (1881-1884) was the first to observe the ability of carotenoids to function as accessory pigments in photosynthesis. Since then, a large number of observations have supported the view that light absorbed by carotenoids can be active in photosynthesis. Fucoxanthin in diatoms (Dutton and Manning, 1941) is as effective as chlorophyll, whereas absorption by carotenoids in *Chlorella* is only partially active (Emerson and Lewis, 1943), and carotenoids in blue-green algae are very ineffective (Emerson and Lewis, 1942).

Similar variations were found in studies on the efficiency of carotenoids as accessory pigments in photosynthetic bacteria; it is thought that the ability of carotenoids to elicit a photosynthetic response is due to their ability to transfer radiant energy to a chlorophyll (Blinks, 1964).

1.5.2. Function in oxygen evolution and transport

The discovery of the widespread distribution of epoxide carotenoids in both photosynthetic and non-photosynthetic organisms (Karrer, 1948) suggested that these forms may function as intermediates in oxygen transport. The observation by Sapozhnikov and Lopatkin (1950) that interconversion of carotenes and xanthophylls can be demonstrated in photosynthetic tissues, led Dorough and Calvin (1951) to suggest that epoxide carotenoids might generate either O_2 or H_2O_2 during photosynthesis and serve as intermediates in the pathway of oxygen evolution. Studies on green leaves and ripening fruit suggested to Cholnoky *et al.* (1956) that the epoxide carotenoids function as intermediates in O_2 transport, although experiments by Yamamoto *et al.* (1962a,b) and Shneour

and Calvin (1962) did not support the view that carotenoids function either in the liberation or in the transport of O_2. Komissarow (1967), who attempted to demonstrate that a photobattery composed of alternate layers of chlorophyll and carotene is capable of liberating O_2 from water under the influence of light, has apparently been unable to substantiate this hypothesis.

1.5.3. Photoreception and phototropism

A number of biological phenomena appear to be initiated or dependent on illumination with blue light. In many of these blue light effects, carotenoids have been found to act as the photoreceptor pigments (Brünning, 1937). However, with the single exception of bacterial phototaxis, the evidence that carotenoids are involved as photoreceptors is inadequate, the most contraversial area being that of phototropism. Evidence that photobiological receptors of phototaxis and phototropism of various organisms involve both flavins and carotenoids, led to the proposal by Briggs (1963) that a transfer of energy from flavins absorbing in the ultraviolet to carotenoids absorbing in the visible region of the spectrum occurs by means of resonance transfer. Recent observations by Pill-Soon Song (1974, 1978),who re-examined photophysical and photochemical considerations of the excited states of flavins and carotenoids, still favours the former as the primary photoreceptor, although the action spectrum for phototaxis in dinoflagellates suggests that carotenoid may be a secondary (antenna) photoreceptor.

1.5.4. Photoprotection

The protective action of carotenoid pigments against lethal photooxidation has been studied in both photosynthetic and non-photosynthetic organisms. The first convincing demonstration of the protective function of carotenoids against chlorophyll photosensitisation has emerged from studies with photosynthetic bacteria by Stanier and his colleagues (Griffith *et al.* 1955).

The non-sulphur purple bacterium *Rhodopseudomonas spheroides* normally grows under anaerobic conditions, but can also tolerate air. A green mutant, devoid of coloured carotenoids, will photosynthesise normally under anaerobic conditions, or grow heterotrophically in an atmosphere containing oxygen in the dark. When, however, the organism is exposed to both light and oxygen for 12-14 hours, massive killing occurs. The same effect was produced by growing the photosynthetic bacterium *Rhodospirillum rubrum* in the presence of diphenylamine, thus inhibiting the production of coloured carotenoids without effecting bacteriochlorophyll synthesis. When diphenylamine-cultured cells were washed to remove the inhibitor and illuminated anaerobically, coloured carotenoids were gradually synthesised leading to a simultaneous reduction in photosensitivity. In each case action spectra indicated that bateriochlorophyll was the photosensitizer.

1.6. The mechanism of photodynamic action

This suggested to the authors that they were observing a photodynamic action defined by Blum (1941) as "the sensitisation of a biological system to light by a substance which serves as light absorber for photochemical reactions in which molecular oxygen takes part." In view of the nature of the action spectra, they concluded that the light absorber sensitising the entire process was the cell's own bacteriochlorophyll. Their experiments supported the view of Griffith *et al.* (1955) that carotenoids are universally associated with photosynthetic tissue because they are apparently capable of protecting cells from photodynamic destruction catalysed by either chlorophyll or bacteriochlorophyll.

In non-photosynthetic organisms, the possibility of oxidative damage by chlorophyll does not exist, but chemotrophic organisms do contain endogenous photosensitizers such as metalloporphyrins and flavins, with an intrinsic ability to catalyse photo-oxidation (Burchard and Dworkin, 1966;

Burchard *et al.*, 1966), thus leading to a sensitisation of the cell to visible light. This phenomenon has been observed in many non-photosynthetic organisms (e.g. *Corynebacterium poinsettiae*, Kunisawa and Stanier, 1958; *Sarcina lutea*, Mathews and Sistrom, 1959b; *Mycobacterium* sp., Mathews, 1963; Wright and Pilling,1963) and mutant strains of algae, (*Chlorella vulgaris*, Claes, 1954; *Chlamydomonas reinhardi*, Sager and Zalokar, (1958) where severe carotenoid deficiencies led to death when aerobic cultures were exposed to light. A protective function under these conditions might explain the occurrence of carotenoids in many bacteria found in habitats exposed to sunlight, the light-stimulated carotenoid production by both bacteria (*Mycobacterium* sp.) and fungi (*Neurospora, Pycomyces*), the high concentration of carotenoids in reproductive cells liable to intensive light exposure (e.g. the conidia of *Neurospora* and other fungi (Carlile, 1965) or in the eggs of many invertebrate species (Cheesman *et al.*, 1967), where they appear to play a role in development). The observation that photosensitizers (metalloporphyrins) are synthesized throughout development (Helm, 1977) suggests that protection of the embryo from photodynamic action may be one of the functions of carotenoids.

Investigation of the effect of temperature on the rate of photodynamic killing of a carotenoid-less mutant of *Rhodospirillum spheroides* by Dworkin (1958) and a similar mutant of *Sarcina lutea* by Mathews and Sistrom (1960) indicated a temperature coefficient (Q_{10}) close to unity, confirming that cell death was caused directly by a photochemical reaction. Since the original observation by Stanier and his colleagues (Griffith *et al.*, 1955), the protective function of carotenoids has been firmly established in systems that are subject to true photodynamic actions as defined by Blum (1941), involving the simultaneous interaction of visible light, a photosensitising chromophore and O_2. However, the

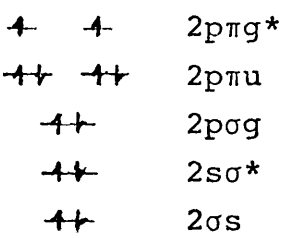
exact molecular mechanism involved in this process is still unknown.

1.6.1. Mechanism accounting for oxygen toxicity in biological systems.

In recent years evidence has accumulated that oxygen is potentially harmful at all concentrations, and that aerobic organisms have evolved protective mechanisms against it.

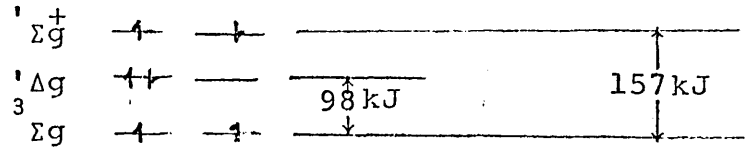
Oxygen is not toxic because of its own reactivity, which is relatively feeble. It has, however, been shown that intermediates in the reduction pathway from molecular oxygen to its final reduction product, water, are exceedingly reactive and must be considered the cause of oxygen toxicity.

Atomic oxygen has the electronic configuration $(1s^2(2s)^2(2p)^4$ In dioxygen the O-O bond may be thought of as one σ bond and one π bond. In the ground state, the 2pσg and 2pπu bonding orbitals are fully occupied with two additional electrons in the degenerate 2pπg* anti-bonding orbitals.



Qualitative molecular orbital scheme for molecular oxygen

The two unpaired electrons of parallel spin resulting from such occupancy are characteristic of a triplet energy level and impart paramagnetic properties. These parallel electron spins normally forbid direct entry of paired electrons, i.e. transition from the $^1\Delta g$ or $^1\Sigma g^+$ excited states to the $^3\Sigma g^-$ ground states. Although such transitions do occur, they have extremely low probabilities and the time required for a change of spin state is orders of magnitude higher than the life time of a collisional complex, explaining the relatively long lifetime of singlet oxygen.



π* orbital occupancy and energies of triplet and singlet oxygen.

The net result of this spin restriction is that :

1) Oxygen in the ground state is much less active as an oxidant than one might expect and

2) univalent pathways of oxygen reduction will be favoured over divalent pathways. This propensity for univalent reduction makes the production of radical intermediates a likely occurrence.

Several enzymes and redox couples are involved in oxidation reactions in which a single electron is transferred to oxygen, thus producing a radical known as superoxide :

$$O_2 + e^- \rightarrow O_2^{\cdot-} \qquad (1)$$

Enzymes involved in this process nearly always contain a flavin prosthetic group. $O_2^{\cdot-}$ is also formed during the autoxoidation of quinones, and both spinach and clostridial ferredoxins can be shown to produce superoxide (Fridovich, 1970). $O_2^{\cdot-}$ has a lifetime of the order of milliseconds and in the absnece of anything else that can react with it, it reacts with itself via a second-order dismutation reaction :

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (2)$$

There is evidence that H_2O_2 and $O_2^{\cdot-}$ can react together to produce $OH^{\cdot}$ radicals. Although the reaction pathway is by no means clear, there is evidence that it is catalysed by traces of metal ions found in practically all living tissues (Cohen, 1977). $OH^{\cdot}$ radicals are among the most reactive species known to organic chemistry, since they will attack

almost every molecule found in living tissues (Anbar and Neta, 1967).

There have also been numerous reports of the formation of singlet oxygen in complex systems undergoing autoxidation (Smith and Kulig, 1976) and it seems that part of the oxygen produced during the breakdown of $O_2^{\cdot-}$ radicals is $^1\Delta g$ oxygen which is far more reactive than triplet oxygen. It is able to oxidise many biomolecules and can attack olefinic bonds, thereby initiating lipid peroxidation.

Although few chemical or biochemical reactions of $O_2^{\cdot-}$ itself have been discovered that are of the type generally acknowledged to be deleterious to the living cell, the real danger of the radical seems to lie in its ability to form $OH^{\cdot}$ and singlet oxygen. It is not known whether singlet oxygen can be generated during the spontaneous dismutation reaction or during reactions between H_2O_2 and $O_2^{\cdot-}$. The evidence is based either on the use of chemical trapping agents, presumed to be characteristic of singlet oxygen, or on the observation of weak chemiluminescence thought to be associated with $^1\Delta g$ to $^3\Sigma g^-$ transition. Since the effects observed were attenuated on addition of superoxide dismutases (SOD), i.e. enzymes catalysing reaction (2) (McCord and Fridovich 1968), it was suggested that the uncatalysed dismutation of superoxide ions can give rise to singlet oxygen. Rapid removal of $O_2^{\cdot-}$ is also thought to prevent formation of $OH^{\cdot}$ radicals.

1.6.2. Singlet oxygen and carotenoid protection in vitro

Several mechanisms have been proposed, whereby carotenoids may protect cells from light and O_2. Goldstrohm and Lilly (1965), who worked on the fungus *Dactylopinax spathularia*, suggested that carotenoids might serve as light-filtering pigments since they found that carotenoid filters increased the survival of dark-grown cells when exposed to high light

intensity. Vrail and Lilly (1968) showed, moreover, that the carotenoids in this fungus were present in cell wall fractions where they could act as light filters, screening out potentially harmful radiation.

In 1955, Calvin put forward the idea that carotenoids could function as protective agents by acting as preferred or competitive substrates for the primary agent of photosensitised oxidation, i.e. O_2, which would interact with a suitable acceptor to form the oxidised product, in this case an oxidised carotenoid, and in its absence lead to potentially lethal photodynamic action.

Sistrom *et al.* (1956) suggested that epoxides might be formed across the double bonds in carotenoids. Krinsky (1966) considered that this mechanism, accompanied by dark-regeneration of oxidised carotenoid to its original acceptor state, might explain the protective action of carotenoids. However, recent observations on the quenching *in vitro* of singlet oxygen by carotenoid molecules render this mechanism unlikely.

One of the earliest mechanisms for production of, and protection against singlet excited oxygen was proposed by Kautsky in the 1930's (Kautsky and de Bruijn, 1931; Kautsky, 1939). He proposed that the triplet sensitiser reacted with oxygen to give singlet excited oxygen $'O_2^*$ which then reacted with an acceptor molecule (A)

$$^3SENS + {}^3O_2 \longrightarrow SENS + {}'O_2$$
$$'O_2 + A \longrightarrow AO_2$$

Later, Schenk (1954) proposed that the sensitizer itself, in the triplet form, is oxidised, and that oxygen is transferred to an acceptor molecule to yield the ground state sensitizer :

$$SENS \xrightarrow{h\nu} {}'SENS$$
$$'SENS \longrightarrow {}^3SENS$$

$$^3SENS + O_2 \rightarrow SENS - O_2$$

$$A + SENS - O_2 \rightarrow AO_2 + SENS$$

Foote and Wexler, (1964) and Corey and Taylor (1964) observed that singlet oxygen produces effects analogous to those obtained in photosensitized oxidations. Since singlet oxygen could be involved in various oxygenase reactions in biological systems, it appears, that as such, it would be a reasonable intermediate in photosensitized oxidations (Foote, 1968). In 1968, Foote and Denny looked at the interaction between singlet oxygen and carotenoids. They argued that since the ability of carotenoids to quench triplet sensitizer is a diffusion-controlled reaction it would compete with the similarly controlled quenching of triplet sensitizer by O_2. Since both reactions occur at the same rate, carotenoid protection would only occur if their local concentration greatly exceeded that of O_2. However, their experimental evidence proved the alternative explanation that carotenoids interact with singlet oxygen.

From studies on the inhibition of methylene blue-sensitized oxidation of 2-methyl-2-pentane, Foote *et al.* (1970) suggested the following mechanism for carotenoid and singlet oxygen interaction :

$$^1O_2 + {}^1CAR \longrightarrow {}^3CAR + {}^3O_2$$

The carotene goes into the triplet state, which process must be reversed, since one molecule of β-carotene quenches about 10^2 molecules of singlet oxygen, and for every 10^3 molecules of β-carotene involved in the quenching of singlet oxygen, only one is oxidised.

1.6.3 Photoprotection and chromophore length

Stanier (1959), Crouse *et al.* (1963a, b), and Mathews and Krinsky (1970b) have demonstrated that the ability of carotenoids to provide protection against aerobic photosensitivity *in vivo*

is related to the length of the conjugated chain of double bonds in the carotenoid molecule.

The most efficient photosensitizers, according to Foote (1968), are those with a long-lived triplet state in high quantum yield. This author states that the reaction :

$$^1O_2 + {}^1CAR \rightarrow {}^3CAR + {}^3O_2$$

can only occur when the triplet energy of β-carotene is near or below that of the $^1\Delta g$ of oxygen, which is 94 kJ.

Experiments *in vitro* by Foote *et al.* (1970a) showed a good correlation with observations *in vivo* in that the rate of quenching of singlet oxygen, reflected in the protective action of the carotenoids, is dependent on the length of the conjugated polyene chain, since the rate of singlet oxygen quenching falls off with decreasing chain length, with the sharpest drop between seven and nine conjugated double bonds.

To quench the triplet level of, for example, chlorophyll *a*, which has an energy in the region of 121 kJ, β-carotene must have a lower triplet energy; in order to quench singlet oxygen, the triplet energy must be less than 94 kJ, and it is likely that the value for β-carotene is in this region. If a decrease in the number of double bonds were accompanied by an increase in triplet energy level, this would explain why carotenoids with shorter conjugated double bond systems will not protect. Although the exact values of the triplet states of β-carotene and other carotenoids are still uncertain, the triplet energy of a carotenoid with nine double bonds in conjugation must therefore be in the region of 94 kJ or less, and the triplet energy of one with eight double bonds should be higher.

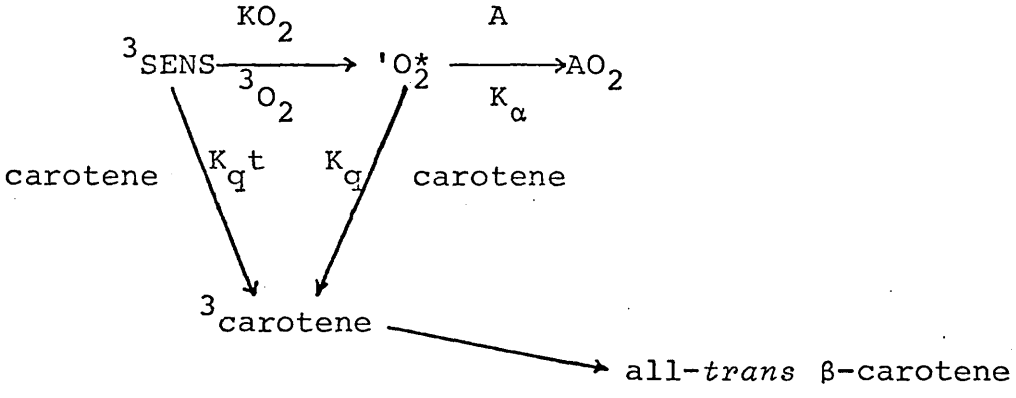
1.6.4 The mechanism of photoprotection

Foote and his collaborators (1968, 1970a,b) have convincingly demonstrated the ability of a variety of carotenoids to

quench the singlet state of oxygen effectively without the pigment suffering chemical change, thereby breaking the series of reactions which would normally lead to photosensitized oxidation.

In 1970, using 15,15'-*cis* β-carotene, they (Foote *et al.* 1970b) found that as a response to exposure to singlet oxygen, this carotenoid was isomerised to the all *trans* form (under the conditions of the experiment, the *trans* β-carotene would not revert to the *cis* form). Each singlet oxygen molecule formed, caused the isomerisation of less than one molecule of *cis* β-carotene to all-*trans* form.

These observations led the authors to propose the scheme outlined below :



Kq^t - rate of quenching of triplet sensitizer (^{3}SENS)

Kq - rate of quenching of singlet oxygen ($'O_2^*$)

The presence or absence of oxygen does not affect the rate of isomerisation, since the rate of quenching of singlet oxygen is much greater than the rate of oxidation of an acceptor.

An alternative mechanism involves reversible electron-transfer from β-carotene to singlet oxygen. This also fits in both with the isomerisation results and with the dependence of quenching efficiency on the number of conjugated double bonds,

since the ionisation potential of carotenoids increases with decreasing conjugation. In addition, the carotene radical cation should isomerise relatively easily, although it might be expected to undergo other reactions as well.

1.7. Conversion of light energy into electrical energy

Carotenoid pigments and the related retinenes are characterised by their ability to absorb light. It has been suggested that, when arranged in a highly ordered fashion, these pigments should allow solid state phenomena such as photoconduction or exciton migration; such a mechanism might play a role in visual excitation (Rosenberg, 1966). The visual pigment is composed of two parts: the chromophore retinal, and the protein moiety opsin, coupled by a Schiff base condensation. The problem of photoconductivity can be resolved into two separate questions :

a) Can the process by which the chromophore becomes excited to its first singlet excited state transmit a mobile electronic charge to its neighbours?

b) Can proteins conduct electric current by movement of electronic charge carriers?

The first phase has been investigated by testing the photoconductivity of β-carotene, i.e. that of a model system of molecules related to the chromophore. When powdered, crystalline β-carotene was sandwiched between two electrodes and irradiated, the current increased, and it could be shown that the photocurrent was linearly related to the intensity of the incident light. When the light was turned off, the current returned to its dark value within seconds. As is generally found for photoconductive processes, the decay of photocurrent has a longer time constant than the rise time. It has been suggested that carotenoid excited to a triplet state is a kinetic intermediate in the process leading to charge carrier production.

The action spectrum for this process shows good agreement with the absorption spectrum of the carotenoid. After light absorption the chromophore is thought to release an electronic charge into the protein molecule and extensive movement of the mobile charge is supported by the protein. As early as 1941, Szent-Györgyi suggested that energy transfer in proteins could occur, since the regular arrangement of peptide linkages in proteins could result in the existence of bands of energy, similar to those present in semiconductors. Results by Arnold and Maclay, (1958), and Rosenberg (1962a) indicate that both crystalline and amorphous proteins are semiconductors.

Although photoconductivity of carotenoid pigments has been clearly established, it remains doubtful whether processes like exciton migration are involved in photorecptor excitation.

1.8. A retinal-protein complex as part of a light-operated hydrogen ion pump coupled to ATP synthesis.

The "salt-loving" halobacterium *Halobium* synthesises purple membrane patches when its normal supply of metabolic energy is interupted. The oxygen consumption by respiring cells is reduced by light when the purple membrane is present (Oesterhelt and Stoeckenius, 1971, 1973). The bacteria can utilise light energy which is first converted into a gradient of hydrogen ions across the membrane, and this electrochemical gradient is then utilised for ATP production to maintain a variety of essential metabolic functions including ion transport. Isolated purple membrane contains 75% protein and 25% lipid. SDS electrophoresis shows that all the protein migrates as a single sharp band, indicating that only one polypeptide of molecular weight 26,000 is present. The chromophore retinal is linked to the protein via a Schiff linkage involving the ε-amino group of one of the seven lysine residues present in the protein. Because of many similarities

with rhodopsin of the visual receptors, the protein was named bacteriorhodopsin. Purple membranes ≈ bacteriorhodopsin are a very stable biological material. The chromophore is not destroyed between pH 1 and 10 and withstands temperatures up to 80°C. Proteinases attack the protein with great difficulty (Oesterhelt, 1976). UV CD spectra of the protein and X-ray diffraction analysis of isolated purple membrane indicate that the protein is coiled into seven closely packed α-helices extending parallel to the long axis of the molecule and roughly perpendicular to the plane of the membrane. The spaces between the protein molecule are filled by lipid bilayer (Henderson and Unwin, 1975).

On illumination, the chromophore, which initially absorbs at 580 nm, undergoes a bathochromic shift to 590 nm, suggesting an increase in conjugation before transient bleaching with a shift to 412 nm occurs. The formation and decay of the 412 nm complex, which is the longest lived intermediate of the cycle, are accompanied by proton release and uptake. These occur as a vectorial process in the purple membrane, i.e. protons released from one side of the membrane are taken up by the other side, resulting in an electrochemical gradient between inside and outside (Oesterhelt and Hess, 1973).

Resonance Raman spectroscopy indicates that in both the purple membrane complex (λ_{max}570 nm) and the first intermediate (λ_{max}590 nm) the Schiff base retinylidene-lysine linkage is protonated, while in the 412 nm complex it is unprotonated. Flash spectroscopic results show that the release of a proton is most closely related to the formation of the 412 nm complex, indicating that the Schiff base may be directely involved in proton release. (Lewis *et al.* 1974).

It is believed that the proton is released from the external surface of the isolated purple membrane and subsequent proton uptake occurs from

the cytoplasmic side, since "right side out" vesicles of reconstituted purple membrane show a net alkalinisation within 10 msec of illumination, whereas "inside out" vesicles show a net acidification (Lozier *et al.* 1976).

The electrochemical gradient of hydrogen ions is thought to be utilised for ATP synthesis in accordance with the chemi-osmotic theory of Mitchell (1966). The action spectra of light-induced pH changes, inhibition of respiration by light, and ATP synthesis follow the absorption spectrum of isolated purple membrane, indicating these these effects are initiated by the absorption of light by the chromophore. Thus, the function of bacteriorhodopsin as a light-driven proton pump seems to be proven beyond doubt.

1.9. Carotenoids in electron transport.

It has been suggested that carotenoids should be both excellent electron donors and electron acceptors (Pullman and Pullman, 1963; Dingle and Lucy, 1965; Platt 1959). The values for the energy coefficients of their highest occupied and lowest empty molecular orbitals certainly support this view. Conjugation to protein should enhance this ability, especially in view of the semiconductor capacity of proteins (Szent-Györgyi, 1941; Arnold and Maclay, 1958; Rosenberg, 1962a).

As yet, there is only suggestive evidence to support the theoretically sound concept of an involvement of carotenoids or carotenoproteins in electron transport. This has been obtained mainly from studies *in vivo* of molluscan neurones.

1.9.1. Carotenoids in oxidative metabolism

A strong yellow or orange-red pigmentation in the nervous system of many snails and bivalves has been observed since the 19th century (Schulze, 1879; Rawitz, 1887). More recent reports indicate that the pigment is localised intra-cellularly in specialised granules. Nolte *et al.* (1965)

called these granules cytosomes, a term which appears to be now generally accepted. These organelles are characterised by a high concentration of carotenoids and a low concentration of myoglobin. Nolte and her coworkers (1965) and Zs.-Nagy (1967) reported that specific mitochondrial enzymes such as succinate dehydrogenase and cytochrome oxidase are also localised in these granules.

Karnaukhov (1971) confirmed the existence of a mitochondrial cytochrome *c*/cytochrome oxidase system in the ganglia of *Lymnaea stagnalis*. He showed that under conditions of oxygen depletion, inhibition of cytochrome oxidase by e.g. KCN, or increase in cell activity, a reversible increase of the carotenoid absorption bands at 465 and 495 nm could be observed. He suggests that a reversible change in the number of conjugated unsaturated double bonds may produce the observed difference in the absorption bands, and believes that the apparent role of carotenoids in oxidative metabolism is to provide a large number of unsaturated double bonds as the site of an intracellular oxygen reserve. Under conditions of low metabolic activity, an excess of oxygen is stored by the carotenoid double bonds. This leads to a decrease in the number of double bonds, with a decrease of absorbance at 465 and 495 nm. Myoglobin is said to serve as an "oxidase" for unsaturated double bonds. Under conditions of high metabolic energy or when oxygen diffusion is inadequate, withdrawal of oxygen from the store will take place, leading to an increase in the number of conjugated double bonds characterised by the observed increase in absorption. Inhibition of the terminal oxidase by KCN leads to withdrawal of oxygen from the store, whereas amytal does not produce a similar effect. The oxygen store appears to be connected with the respiratory chain in the region of the flavoprotein. Although Karnaukhov finds it difficult to speculate about the mode of binding of oxygen with carotenoids, he considers the possibility that the latter

serve as "oxygenases" (sic) by forming either unstable peroxides or aldehydes with oxygen.

1.9.2 Cytosomes in anoxic energy production in molluscan neurones.

Certain snails and bivalves are able to survive surprisingly long periods of anoxia (Brand *et al.* 1950). The nature of the anoxic energy production, is however, not clear. Zs.-Nagy (1967 , 1970) showed that the anoxic tolerance of molluscs is connected with the presence of the above mentioned pigment granules or cytosomes (Nolte, 1965) in the nervous and other tissue. He compared the survival time of 11 different molluscs and obtained a remarkably well defined correlation between the degree of pigmentation and anoxic tolerance. The highest tolerance was shown by the bivalve *Anodonta cygnea* (7-10 days), whereas other less pigmented species show shorter survival times, and those with an unpigmented central nervous system do not tolerate anoxia at all. The term anoxic tolerance always refers to gradually created anoxia. If animals are placed in water initially devoid of oxygen, even those of otherwise high anoxic tolerance die quickly (Zs.-Nagy and Ermini, 1972b; Zs.-Nagy, 1974). This indicates that the mechanism protecting the animals against anoxia is subject to gradual adaptation. Since, under natural conditions, gradual development of anoxia is more probable than immediate development, anoxic tolerance is considered an adaptation to the environment.

The functional significance of cytosomes appears, on the above evidence, to be related to anoxic energy production in molluscs with nervous systems rich in these organelles. Respiratory enzyme activity similar to that obtained in mitochondria appears to play an important role in cytosomal functions, indicating the possibility of some connection between mitochondria and cytosomes (Fährmann, 1961). Morphological changes in cytosomes during anoxia and those in

mitochondria during reoxygenation suggest a possible transformation of mitochondria into cytosomes (Zs.-Nagy, 1969). This view is further supported by the energy-dependent accumulation of divalent cations in cytosomes, with the cytosomal mechanism becoming activated only during prolonged anoxia. The accumulation remains sensitive to KCN and DNP in the complete absence of molecular oxygen. The changes in cytosomes during anoxia have been interpreted by assuming that cytosomes are at rest during normal aerobic respiration and become activated by decreasing oxygen tension (Zs.-Nagy, 1971a; Kerpel-Fronius and Zs.-Nagy, 1973).

1.9.3 Electron acceptor properties of the lipochrome pigment

Since the lipochrome pigment of cytosomes contains carotenoid, and in view of the already mentioned observations by Karnaukhov (1971) which suggested that the electron distribution in the pigmented region changes in anoxia, Zs.-Nagy (1971b) investigated the possibility that the pigment complex may represent some kind of electron acceptor. The experiments were carried out with isolated ganglia of *Anodonta cygnea* and *Lymnaea stagnalis*. Frog brain was used as a control poikilothermic nervous tissue devoid of lipochrome pigment. The behaviour of the redox potential during these experiments strongly supported the view that the nervous tissue of mollusc contains an electron acceptor which protects tissue components from reduction even during anoxia and which is absent in frog brain.

The lipochrome pigment has a redox potential in the region of $E_m \simeq 350\text{-}400$ mV, which is lower than that of O_2 ($E_m \simeq 820$ mV), but higher than that of cytochrome *a* ($E_m \simeq 290$ mV) which therefore should be oxidised by the lipochrome pigment. Therefore, by means of anoxic endogenous energy production, about 2/3 of the normal ATP level may be produced (Zs.-Nagy, 1971b). Zs.-Nagy and Ermini (1972a) also showed that ethanol

or 2-propanol extracts of *Mytilus galloprovincialis* contain a component or components able to oxidise NADH. The oxidative effect of the extracts depends on the concentration and has been shown to be of a reversible nature. Molecular oxygen does not participate in this oxidation. Since it is difficult to imagine that, in the presence of about 80% ethanol or 2-propanol, any enzymic process can persist, a direct chemical oxidation has been suggested.

The question as to the nature of the components responsible for the oxidative effect has not yet been resolved, since the lipochrome fraction is of rather complex composition, containing carotenoids, neutral and acid lipids and phospholipids, and possibly flavins and quinones and other factors not yet identified.

Another interesting aspect is that the oxidative capacity of extracts prepared from animals kept under prolonged anoxic conditions is greatly diminished. This clearly indicates that the absolute amount of oxidant decreases during anoxia.

As already mentioned, the conclusion that cytosomes play a role in anoxic energy production was also reached by Karnaukhov, who assumed that carotenoid could bind oxygen by means of its conjugated double bond system thus forming an oxygen store. However, there is no evidence that lipochrome pigments including the carotenoid are capable of binding oxygen (Pearse, 1972).

Quantitative analysis by Zs.-Nagy (1974) showed that, even if oxygen storage took place according to the assumption of Karnaukhov and if one mole of carotenoid could bind 5 moles of oxygen, the amount of carotenoid present in *A. cygnea* ganglia is too small (by a factor of 10^5) to provide the oxygen demand during the observed anoxic survival time of some 150-240 hours. He also indicated that if a combination of carotenoid, haemoglobin, myoglobin and haemocyanin were

involved in some way in oxygen storage, the quantities of these substances would not even account for one per cent of the total oxygen demand during 150 hours. On the basis of these observations, Zs.-Nagy (1974, 1976) rules out the possibility that any form of stored oxygen is involved in anoxic endogenous oxidation, and presents evidence that the internal electron acceptor function replacing molecular oxygen is performed by unsaturated fatty acids.

These results render some features of the very attractive hypothesis of Karnaukhov (1971), that carotenoids are involved directly in oxidative metabolism, somewhat doubtful; they do not, however, exclude a catalytic function of these substances in oxidative pathways.

1.10. The purpose of the present investigation

The investigation was initially designed to give information about the molecular properties and the biological significance of the two chromoproteins in the egg jelly of *Pomacea canaliculata*, ovorubin and helicorubin P.

A considerable time was devoted to the molecular properties. The work related to the"biological significance"was largely concerned with a suggestive interrelationship of the two proteins which came to light at an early stage of the experiment.

Preliminary experimental evidence had shown that removal of ovorubin from egg homogenates of *P. canaliculata* led to rapid oxidation of helicorubin which is normally 70-95% in the reduced state. This indicated that :

a) the presence of ovorubin influences the redox state of helicorubin and
b) a helicorubin oxidase is present in the egg homogenate.

It was decided to investigate the possibility that ovorubin and helicorubin form part of an extracellular electron transport system.

Factors affecting the interaction of ovorubin and helicorubin in both the homogenate and in the isolated state were investigated by applying some of the experimental methods employed in studies of oxidative phosphorylation and photophosphorylation. In particular, a search was made for a possible function of the carotenoid moiety of ovorubin in these interactions.

CHAPTER 2

THE INTERACTION OF OVORUBIN AND HELICORUBIN IN HOMOGENATES FROM THE EGGS OF *POMACEA CANALICULATA*

2.1 Introduction

Preliminary studies showed that removal of ovorubin by adsorption on Con A-Sepharose (for experimental details, see Chapter 3.2.) led to a progressive oxidation of helicorubin, which in freshly laid eggs (collected within *ca.* 14 hrs of laying and stored for up to 18 months in a deep freeze at -20°C) is between 70-95% in the reduced state. The increase in the degree of oxidation of helicorubin after removal of ovorubin is less pronounced when the homogenate is centrifuged at 10,000g than at 2,000g. This suggests the presence of a particulate helicorubin oxidase, and a function of ovorubin in maintaining helicorubin in a reduced state.

Early observations also showed that the nature of the buffer system affected the degree of reduction of helicorubin in the homogenate. In a Na phosphate buffer system at pH 7.0, a pH at which helicorubin is not auto-oxidisable, it was invariably more reduced than in water at the same pH, while in a Tris-maleate buffer at pH 7.0, a further reduction could occasionally be observed. Since 0.1 M Tris-HCl did not affect the redox state of helicorubin in a detectable degree, this buffer was chosen for the experiments. Homogenates freed from ovorubin (see 2.2. 2) were not influenced in a comparable fashion by the nature of the buffer system.

Difficulties were initially encountered in obtaining preparations of ovorubin spectrophotometrically and electrophoretically free of helicorubin. This indicated a close interaction between these proteins, which was further suggested by the finding that, when attempts were made to purify ovorubin from egg homogenates by micropreparative electrophoresis (see Chapter 3.2.) without prior purification of the protein by affinity chromatography or by the method of Cheesman (3.2.), all the fractions obtained on electrophoresis at pH 8.3 were contaminated with helicorubin.

These observations initially gave rise to the erroneous conclusion that helicorubin from *P. canaliculata*, like that from *H. pomatia*, is an acidic protein (Keilin, 1957, 1968). Further

experimentation (3.3) showed, however, that it is a basic protein. In the absence of ovorubin, helicorubin does not migrate towards the anode at pH 8.3, but moves towards the cathode at pH 4.5. It does not co-purify with the cathodic fractions of ovorubin, which are completely free from it under otherwise comparable experimental conditions (pH 4.5).

These preliminary observations were consistent with an involvement of these two proteins in electron transport in the egg jelly, which represent at least 60% of the total protein. It was therefore decided to apply criteria used in studies of mitochondrial oxidative phosphorylation to the egg jelly system.

2.2. Methods and materials

Ascorbic acid, reduced glutathione, KCN, Na dithionite, dithiothreitol, Na persulphate, Na azide, Na arsenate, NADH, NADPH and KH_2PO_4 were all obtained from BDH Ltd.

Initial experiments were carried out with ATP from Kyowa-Hakko-Kogyo Co. Ltd. (Tokyo). Subsequently ATP, ADP and AMP were purchased either from BDH Ltd., Sigma Chemical Co., or Boehringer, (Mannheim).

Antimycin, oligomycin, heptylquinoline oxide and rotenone were provided by Sigma Chemical Co., while Na amytal was obtained from Eli Lilly and Co.

α, β-methylene-adenosine 5'-diphosphate was supplied by Miles Laboratories Inc., while P^1P^5-di adenosine 5'-pentaphosphate (Ap_5A) was obtained from Boehringer.

The firefly lantern extract was purchased from Sigma Chemical Co.

All experiments were carried out at 30°C and pH 7.0. This pH was chosen since unbuffered aqueous homogenates of *P. canaliculata* were in the pH range between 6.95 and 7.05.

2.2.1. Preparation of egg homogenates

Initially, these were prepared from eggs collected by Miss M. Walter in the marshes of Paraguay. They were at intermediate stages of development; some eggs probably derived from hybrid species of *P. canaliculata* and *P. insularum*. During

the later stages of the investigation, homogenates were prepared from the eggs of *P. canaliculata* collected at times from 30 min. to six days after laying and used immediately or stored at -20°C for up to 18 months. When eggs stored in the freezer were used, they were allowed to thaw at 4°C and subsequently homogenised in 0.1 M Tris-HCl buffer pH 7.0 either with a Potter-Elvehjem homogeniser or with a Waring blendor to give a 25% (w/v) homogenate. This was subsequently centrifuged at 2,000g for 20-30 min., or at 35,000g and 85,000g in the presence of 10 mM $MgCl_2$ at 4°C in a MSE Superspeed 40 centrifuge. All homogenates used in experiments were diluted to 5% (w/v) unless stated otherwise.

2.2.2. Homogenates freed from ovorubin

25% (w/v) egg homogenates were treated with a Con A-Sepharose gel slurry (see 3.2.1.). While ovorubin (and other substances interacting with Con A) adhered to the bound lectin, the remainder of the homogenate could be filtered off by vacuum filtration. 5% (w/v) homogenates freed from ovorubin were investigated in the same way as the complete homogenates. Reconstituted homogenates were prepared by the addition of 1.4 mg of freeze-dried ovorubin to each ml of 5% homogenate previously freed from it. Ovorubin was purified as outlined in 3.2.1.

2.2.3. Determination of the redox state of helicorubin in the egg homogenate

The initial degree of reduction of helicorubin was determined as follows: Measurements were made of the difference in absorbance between the Soret band of the persulphate-oxidised haemoprotein at 416 nm ($\varepsilon_{mM} = 91$) and that of the dithionite-reduced form at 428 nm ($\varepsilon_{mM} = 147$). The corresponding difference for the untreated homogenate could then be used to calculate the degree of reduction by simple proportion.

The helicorubin concentration in a 5% (w/v) egg homogenate is in the region of 4 μM.

2.2.4. The effect of oxygen perfusion on the homogenate

O_2 (95% + 5% CO_2) was obtained from B.O.C. Ltd., and was bubbled through the homogenate, buffered at pH 7.0 in Tris-HCl,

by means of a Pasteur pipette.

2.2.5. Anaerobic conditions

The effect of anaerobiosis was measured by evacuating air from the homogenate in Thunberg cuvettes (Helma No. 193).

2.2.6. The effect of carbon monoxide

In the early experiments CO was produced by adding concentrated H_2SO_4 to Na formate. The gas was freed from oxygen by passage through an alkaline solution of K pyrogallate. On later occasions it was obtained in cylinders from B.O.C. Ltd., but still passed through alkaline K pyrogallate.

2.2.7. The effect of light on the redox state of helicorubin in the homogenate

Whole homogenates and those freed of ovorubin were exposed to diffuse light (5 W m^{-2}) at 30°C. Appropriate controls were kept under identical conditions in blackened tubes wrapped in aluminium foil. After 24 hrs the redox state of helicorubin in these homogenates was determined by difference spectrophotometry in the usual way. Dark-incubated homogenates were removed from tubes by means of a blackened syringe and transferred immediately to a cuvette in the spectrophotometer.

2.2.8. The action of electron transport inhibitors on the redox state of helicorubin in the egg homogenate in the presence and absence of oxidisable substrates

The effects of NADH, NADPH, GSH and ascorbic acid on the reduction of helicorubin in a 5% (w/v) egg homogenate, or a homogenate freed from ovorubin by adsorption on Con A-Sepharose, were measured by difference spectrophotometry. The assay was performed in 1 ml 0.1 M Tris-HCl buffer pH 7.0 in a 1 cm cuvette thermostated at 30°C. The reaction was started on addition of a 0.1 ml aliquot of substrate to give a final concentration varying from 0.062 to 2 mM in the test cell. The progress of the reaction was monitored for 10 min. by measuring the change in difference spectrum in relation to the reference cell, which contained equal concentrations of all reactants apart from the substrate. This had been replaced by an equivalent volume of 1 mM Na dithionite.

The effects of 2 mM Na amytal, 30 μM rotenone, 1 μM antimycin, 2 μM heptylqunoline oxide, 1 mM Na azide and KCN (all final conc.) were measured by replacing 0.1 ml of buffer by an equivalent volume of buffer solution containing the inhibitor in the reference and test cells under otherwise unchanged assay conditions. Antimycin and rotenone were dissolved in a 50% ethanolic buffer solution. All assays in the presence of inhibitors were carried out at 0.1 mM substrate concentration.

Control experiments were also carried out where the effects of the above-mentioned inhibitors in the absence of substrate were investigated by replacing 0.1 ml buffer in the test cell with the same volume containing the inhibitor. When inhibitors made up in ethanolic buffers were used, 0.1 ml of buffer was replaced by the same amount of 50% ethanolic Tris-HCl buffer pH 7.0 in the reference cell.

2.2.9. The effect of adenine nucleotides on the redox state of helicorubin in the homogenate

The effects of ATP, ADP and AMP on the Fe^{3+}/Fe^{2+} ratio of helicorubin in the homogenate were studied on eggs collected within 30 min. to 6 days of laying and also on homogenates of eggs freed from ovorubin. The changes in the redox state of helicorubin were measured by difference spectrophotometry in the manner outlined in 2.2.3. and 2.2.8. The reaction was started by addition of a 0.1 ml aliquot of the neutralised nucleotide in Tris-HCl buffer pH 7.0 containing between 2.5 and 80 mM. The change in the redox state of helicorubin was measured 10 min. after the addition of the nucleotide to the homogenates in the test cell. The reference cell contained the same concentrations of all reactants, apart from the nucleotide which had been replaced by buffer.

2.2.10. The effect of electron transport inhibitors on the change in the redox state of helicorubin in the presence of adenine nuceotides

This was determined as described in 2.2.8. The volume of oxidisable substrate was replaced by the same amount of 10 mM adenine nucleotide. The change in redox state of helicorubin was measured as outlined in 2.2.3.

Control experiments were carried out in the usual way.

2.2.11. The relationship between the Fe^{3+}/Fe^{2+} ratio of helicorubin and the ATP content of the homogenate after addition of ADP + Pi

Spectral changes on addition of 0.1 ml of 10 mM ADP + Pi to 0.9 ml of a 5.5% (w/v) egg homogenate centrifuged either at 2,000g for 20 min. or, in the presence of 10 mM $MgCl_2$, at 85,000g for one hour, were followed by difference spectrophotometry in the usual way (2.2.3.) for up to 10 hrs.

In early experiments, a Tris-HCl buffer system (0.1 M pH 7.0) was used. In later experiments this was replaced by N-2-hydroxyethylpiperazine-N-2-ethanol sulphonic acid (HEPES 40 mM pH 7.0), since Tris ions are known to uncouple photophosphorylation. No significant difference was found.

The same experiment was carried out with otherwise identical reactant concentrations in a total volume of 5 ml. At fixed time intervals, both the control homogenate and the test homogenate were analysed for their change in ATP content by the method of Izzo (undated). 20 µl of solution containing between 10^{-6} - 10^{-12} M ATP was added to a reconstituted firefly lantern extract in 20 mM $MgCl_2$ - 50 mM Na arsenate buffer pH 7.4 containing 50 µM Ap_5A, a potent myokinase inhibitor, and 50 µM α,β-CH_2-ADP, a creatine kinase inhibitor, since these two enzymes interfering with the assay are found as impurities in the lantern extract. The mixture was stirred thoroughly and the light output followed with an Aminco 'Chem Glow' photometer and chart recorder. The emission at 30 sec. after mixing was taken as a measure of the ATP concentration. Standards were run on every occasion.

Depending on the commercial source, ADP was found to contain between 0.6 and 3% (w/w) ATP, while the myokinase inhibitor contained 0.25% (w/w) ATP.

Control experiments were carried out during which both the control homogenate and the test homogenate were preincubated for 5 min. in the presence of 50 µM Ap_5A to inhibit a potential endogenous myokinase activity.

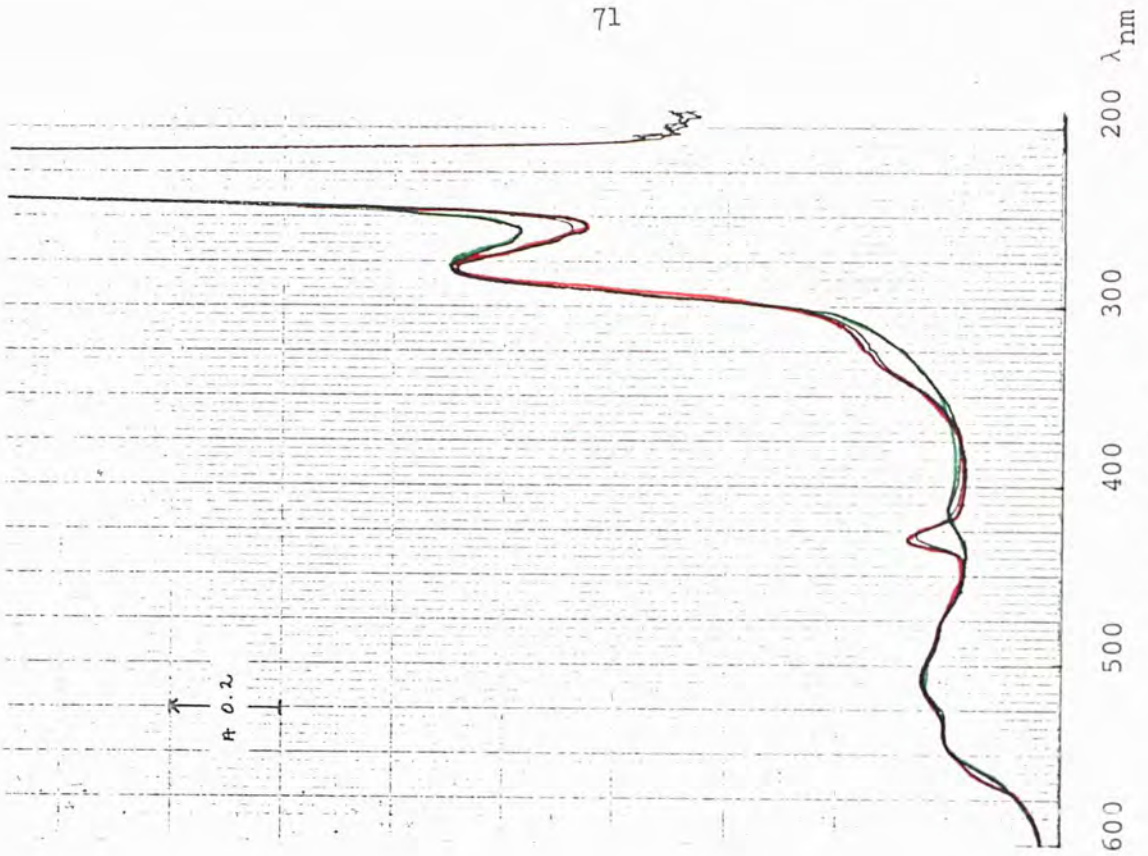


Fig. 2.3.1.a

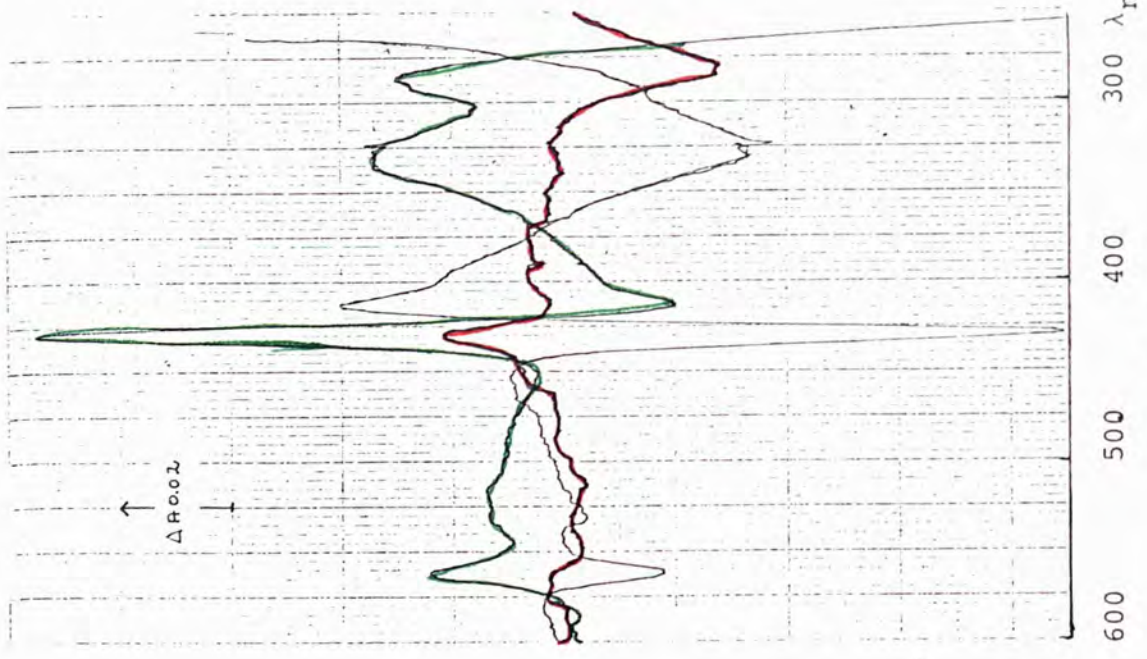


Fig. 2.3.1.b

2.3. Results

2.3.1. Spectral characteristics of the egg homogenate and the effects of O_2, CO, anaerobiosis and light

Fig. 2.3.1.a Spectra of a 5% (w/v) egg homogenate of *P. canaliculata* in 0.1 M Tris-HCl buffer, pH 7.0, path length 0.4375 cm

- homogenate in buffer
- " reduced with 1 mM Na dithionite
- " oxidised with 1 mM Na persulphate

Fig. 2.3.1.b Difference spectra of the above (Fig. 2.3.1.a)

- untreated v. reduced homogenate
- untreated v. oxidised homogenate
- oxidised v. reduced homogenate

Helicorubin was 87% reduced in the untreated homogenate.

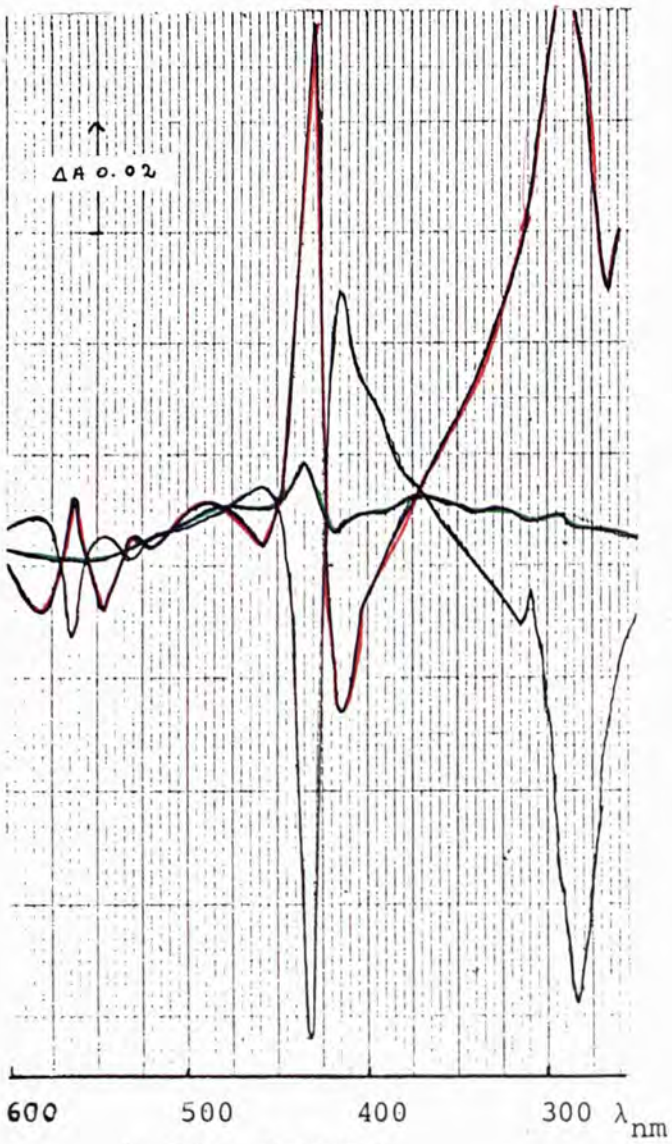


Fig. 2.3.2.b

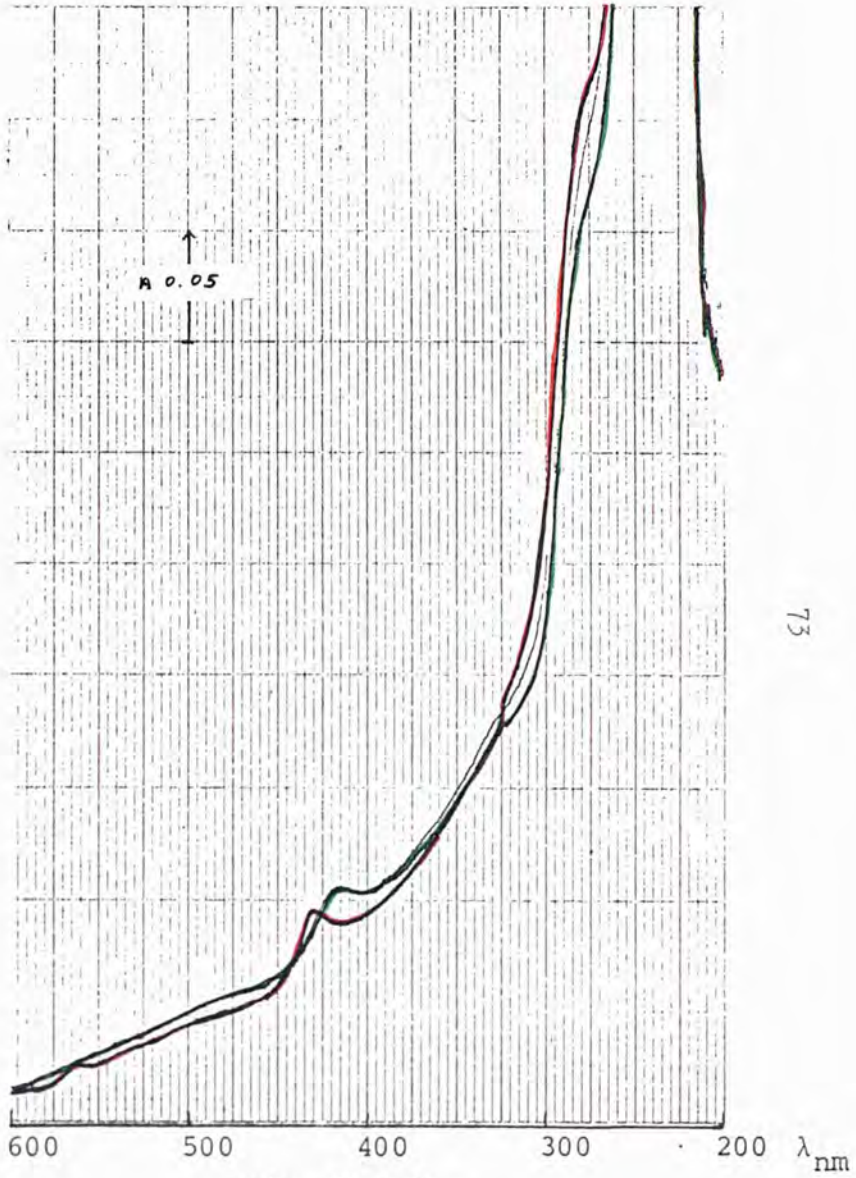


Fig. 2.3.2.a

Fig. 2.3.2.a Spectra of a 5% (w/v) egg homogenate of *P. canaliculata* in 0.1 M Tris-HCl buffer, pH 7.0 after removal of ovorubin by adsorption on Con A-Sepharose gel.

- homogenate freed of ovorubin in buffer
- the same, reduced with 1 mM Na dithionite
- the same, oxidised with 1 mM Na persulphate

Fig. 2.3.2.b Difference spectra of the above (2.3.2.a).

- untreated v. reduced homogenate
- untreated v. oxidised "
- oxidised v. reduced "

Helicorubin was 18% reduced in the untreated homogenate.

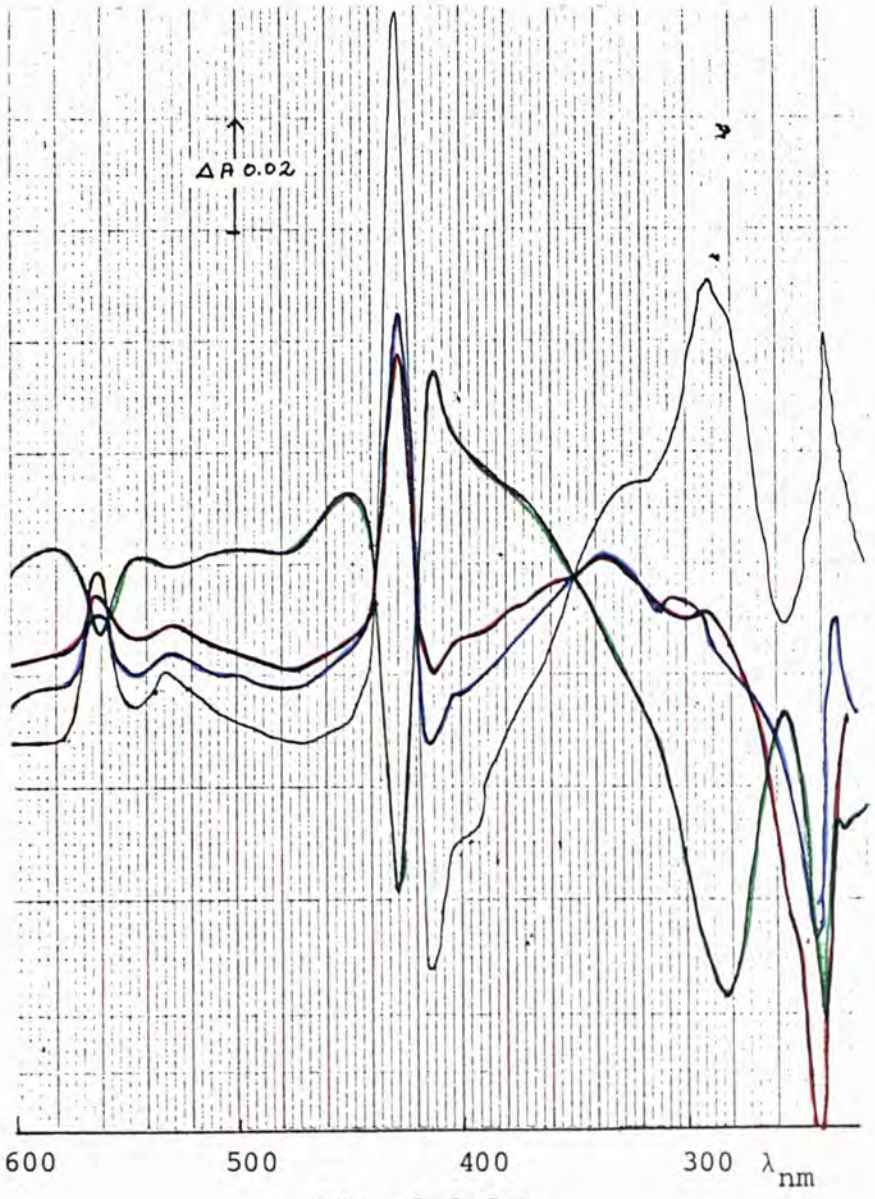


Fig. 2.3.3.b

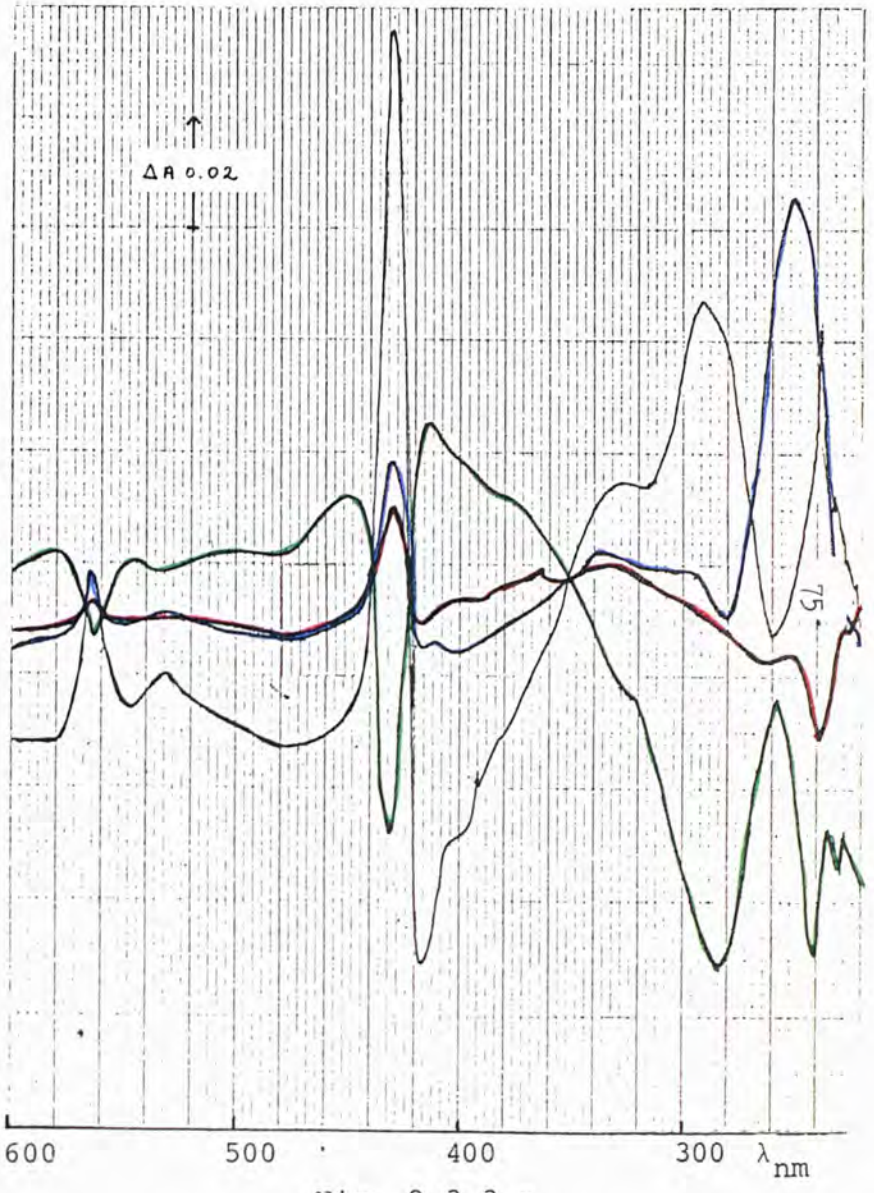


Fig. 2.3.3.a

Fig. 2.3.3.a Effects of oxygen and anaerobiosis on the redox state of helicorubin in the homogenate, pH 7.0. The spectra shown are the difference spectra of:

a) ● reduced v. oxidised homogenate
b) ● reduced homogenate v. deaerated homogenate (5 min.)
c) ● oxygenated homogenate v. reduced homogenate (5 min.)
d) ● reduced homogenate v. untreated homogenate.

The homogenates were prepared in the usual way.

It can be calculated from (a) and (d) that helicorubin was initially 80% reduced. Removal of air led to a further 8% reduction. Oxygenation lowered the level of reduction to 56%.

Fig. 2.3.3.b Effects of oxygen and anaerobiosis on the redox state of helicorubin in a dialysed homogenate. The spectra shown are the difference spectra of:

a) ● reduced v. oxidised homogenate
b) ● reduced homogenate v. deaerated homogenate (5 min.)
c) ● oxygenated homogenate v. reduced homogenate (5 min.)
d) ● reduced homogenate v. untreated homogenate.

For this experiment a 10% (w/v) homogenate was dialysed for 12 hrs against 25 ℓ distilled water at 4°C. Before the start of the experiment it was diluted to 5% (w/v) by the addition of 0.2 M Tris-HCl buffer, pH 7.0. From difference spectra (a) and (d) it can be seen that helicorubin is now 55% in the reduced state; oxygenation decreases this further to 48%, while, under anaerobic conditions, the reduction is increased to 67%.

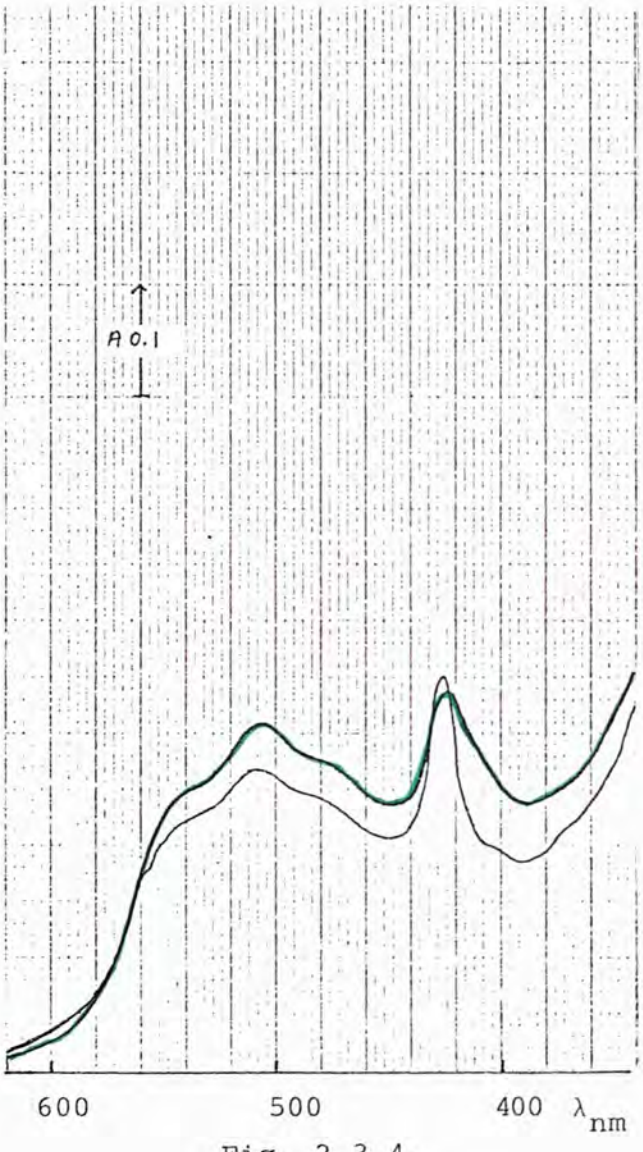


Fig. 2.3.4.

Ovorubin-free preparations of the homogenate continue to show these effects, although they are very greatly reduced, while reconstituted homogenates regain most of the activity of the original system. The effect of oxygenation was usually, however, not very convincing, while that caused by anaerobic conditions was very clear cut.

Under these conditions, reduction of helicorubin was somewhat slower when dialysed homogenates were studied. Control experiments performed on homogenates freed from ovorubin, showed that the reduction of helicorubin after returning ovorubin to the homogenates also occurred in the presence of air. The effect was more pronounced in undialysed homogenates, although dialysed systems showed no qualitative difference from these.

Prolonged deaeration (in excess of 15 min.) leads to the formation of a fine pale yellow precipitate which can be collected after centrifugation at 10,000g for 30 min. Preliminary electrophoretic analysis by SDS-PAGE (see Chapter 3.2.) suggests that this material is the apoprotein of ovorubin which is more unstable and denatures under these conditions.

Extensive aeration of the homogenate leads to an increase in the carotenoid absorption as can be seen from Fig. 2.3.4.

. spectrum of 5% (w/v) homogenate in buffer, pH 7.0

• the same after 30 min. oxygenation.

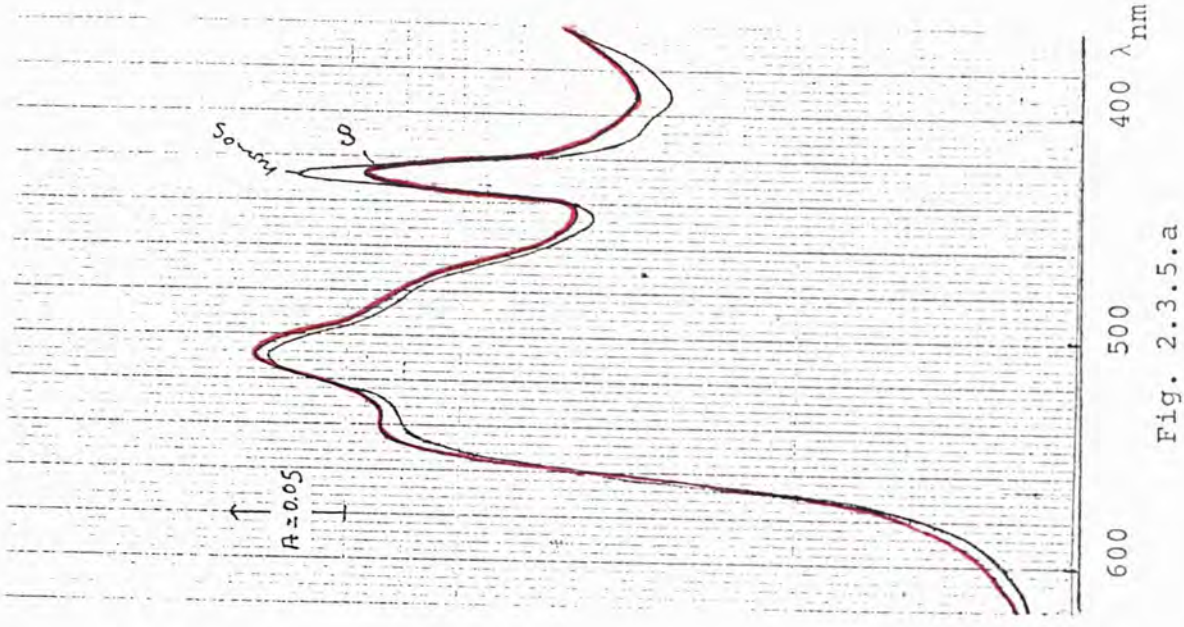


Fig. 2.3.5.a

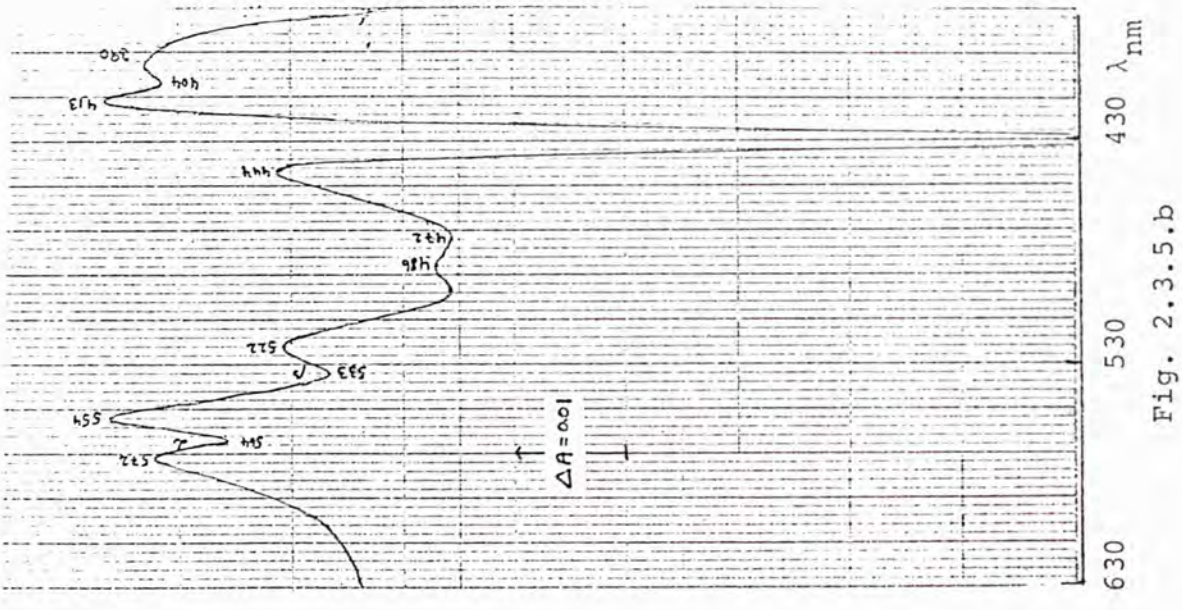


Fig. 2.3.5.b

Fig. 2.3.5.a The effect of carbon monoxide on the homogenate spectrum
- spectrum of a ca. 7% (w/v) egg homogenate in 0.1 M Tris-HCl buffer, pH 7.0
- the same after 5 min. exposure to a steady flow of CO.

Fig. 2.3.5.b Difference spectrum of the homogenate in the reference cell and the CO-treated homogenate in the test cell. The experiments were carried out as described in 2.2.6.

In experiments with carbon monoxide, helicorubin was initially 90% reduced. 10 min. after the initial exposure to CO it was ca. 35% reduced. When this CO-treated homogenate was observed over a period it was found that the maxima and minima of the difference spectrum, which had tentatively been attributed to an effect of the gas on the carotenoid moiety, disappeared within 20 min. Helicorubin became gradually more reduced, until, after 3 hrs, it had almost reached its original value (86% reduced). Homogenates freed from ovorubin by adsorption on Con A-Sepharose do not display CO-binding.

Further experiments showed (Chapter 3) that ovorubin forms a light-labile complex with CO.

	% reduction of helicorubin in homogenate		
	initial	illuminated ($5\ W\ m^{-2}$)	dark
mean	87.4	80.5	77.1
SD	5.9	11.1	11.6
range	95-78	91-62	93-60

Table 2.3.1 The effect of light on the redox state of helicorubin in the homogenate.

All experiments performed at 30°C.
Experimental details in 2.2.7.

The results tabled represent the values obtained in triplicate from five independently prepared homogenates. The initial value represents the degree of reduction of helicorubin in the homogenate at the start of the experiment. On illumination for 24 hrs, there is a decrease of some 7% in the degree of reduction, while in the dark during the same period this value reaches 10.3%. Although it is premature to attribute significance to a mean difference of 3% between the irradiated preparations and those kept in the dark in view of the scatter of the results obtained, the effect of light was further investigated. In Chapter 5.3. results are presented which suggest a light-promoted interaction of ovorubin with cytochromes of the *b* and *c* types.

When ovorubin-free homogenates were investigated, homogenates kept in the dark were always significantly more reduced (11%, mean of 6 experiments) than those exposed to light, which were always completely oxidised.

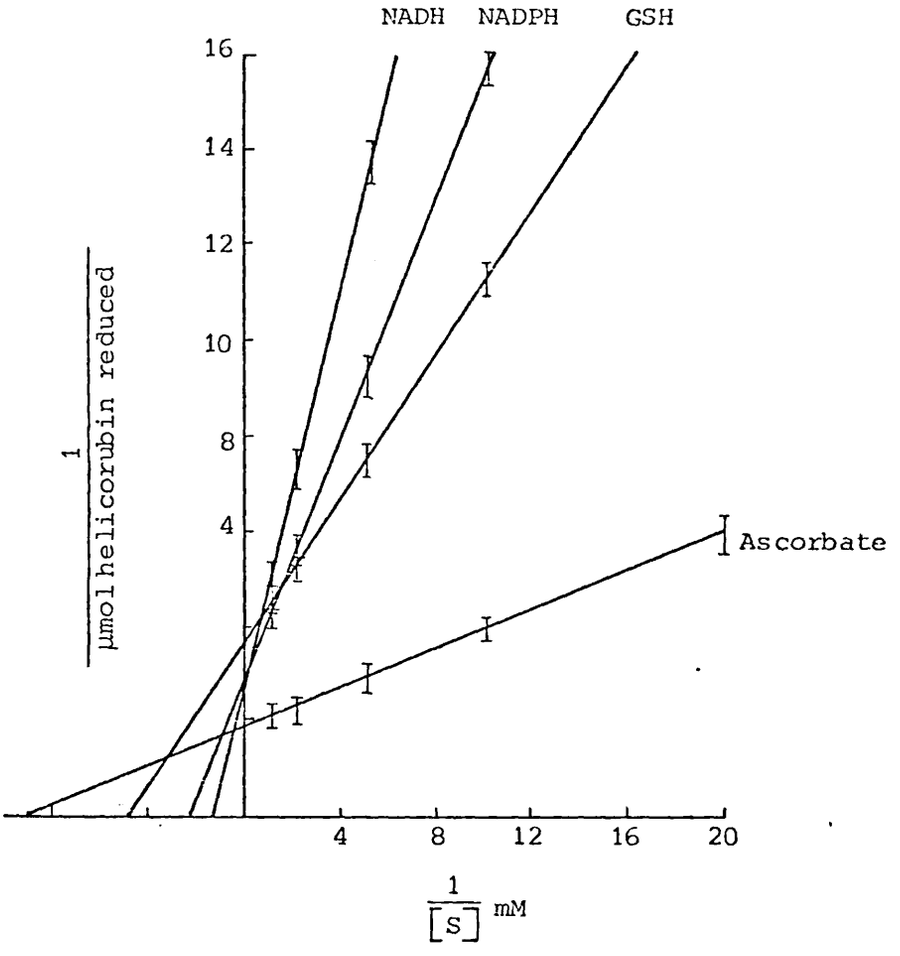

NADH
NADPH
GSH
16
14
12
10
8
4
Ascorbate
1
µmol helicorubin reduced
4
8
12
16
20
1
[S] mM

Fig. 2.3.6

2.3.2. The effect of electron transport inhibitors on the redox state of helicorubin in the egg homogenate in the presence of oxidisable substrates

	redox state of helicorubin
initially	75% reduced
10 min. after addition of 2 mM amytal	80% "
" " " " 30 µM rotenone	81% "
" " " " 2 µM heptyl-quinoline oxide	77% "
" " " " 1 µM antimycin	78% "
" " " " 1 mM Na azide	70% "
" " " " 1 mM KCN	43% "
" " " " 1 µM oligomycin	80% "
" exposure to carbon monoxide	36% "

Table 2.3.2.

The effect of some electron transport inhibitors on the redox state of helicorubin in a 5% (w/v) egg homogenate in 0.1 M Tris-HCl buffer, pH 7.0. (For experimental details see Chapter 2.2.8.) There was little effect when these inhibitors were added to the homogenate freed from ovorubin by its adsorption on Con A-Sepharose.

Fig. 2.3.6. Lineweaver-Burk plots for the reduction of helicorubin in the homogenate in 0.1 M Tris-HCl buffer, pH 7.0.

- ascorbate (K_m = 0.11 mM, V_{max} = 0.53 µmole min^{-1})
- reduced glutathione (K_m = 0.21 mM, V_{max} = 0.27 µmol min^{-1})
- NADH (K_m = 0.71 mM, V_{max} = 0.35 µmol min^{-1})
- NADPH (K_m = 0.46 mM, V_{max} = 0.35 µmol min^{-1})

The results shown represent the readings obtained in triplicate from two different homogenates. The helicorubin in the homogenate at the start of the experiment was between 68 and 73% reduced.

In ovorubin-free homogenates the reduction of helicorubin by NADH, NADPH and GSH was diminished by up to 95%. With ascorbate, reduction levels of about 80% of those found in the presence of ovorubin were achieved.

	Substrate (0.1 mM)			
	NADH	NADPH	GSH	ascorbate
Inhibitor	% inhibition			
2 mM amytal	11	10	4	8
30 µM rotenone	26	20	10	12
2 µM heptylquinoline oxide	11	7	5	3
1 µM antimycin	12	8	3	10
1 mM Na azide	- 5	- 8	- 3	- 3
1 mM KCN	-40	-49	-15	-12
1 µM oligomycin	8	4	3	8

Table 2.3.3.

The effect of the electron transport inhibitors on the reduction of helicorubin in the egg homogenates by oxidisable substrates. (For experimental details, see Methods and Materials 2.2.8).

2.3.3. The relationship between adenine nucleotide concentration and the degree of reduction of helicorubin in the homogenate

	% reduction of helicorubin in homogenate				
Inhibitor		10 min. after addition of 1 mM			
	Control	ATP	ADP	AMP	Pi
uninhibited	73%	51	79.5	80	77
2 mM amytal	78	52	82		
30 µM rotenone	80	48	83		
2 µM heptylquinoline oxide	75	54	81		
1 µM antimycin	75	52	80		
1 mM Na azide	69	55	80		
1 mM KCN	42	66	58		
1 µM oligomycin	79	58	80		

Table 2.3.4.

The change in Fe^{3+}/Fe^{2+} ratio of helicorubin P in a 5% (w/v) egg homogenate on addition of ATP or ADP and the effect of inhibitors on this process.

At the start of the reaction helicorubin was 73% in the reduced state. The progress of the reaction was followed by measuring the change in difference spectrum after addition of ATP or ADP (1 mM final conc.) to the homogenate in the test cell. For further experimental details see 2.2.9 and 10. The figures shown represent the means of four determinations on each of two independently prepared homogenates.

These observations show that the homogenate possesses oxidising and reducing power which can be released on addition of adenine nucleotides. The results in Table 2.3.4. suggest that addition of ATP to the homogenate increases the Fe^{3+}/Fe^{2+} ratio while ADP lowers it.

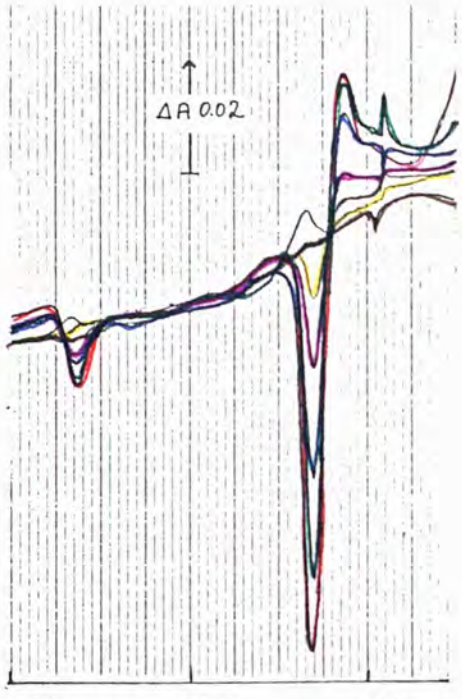


Fig. 2.3.7.a

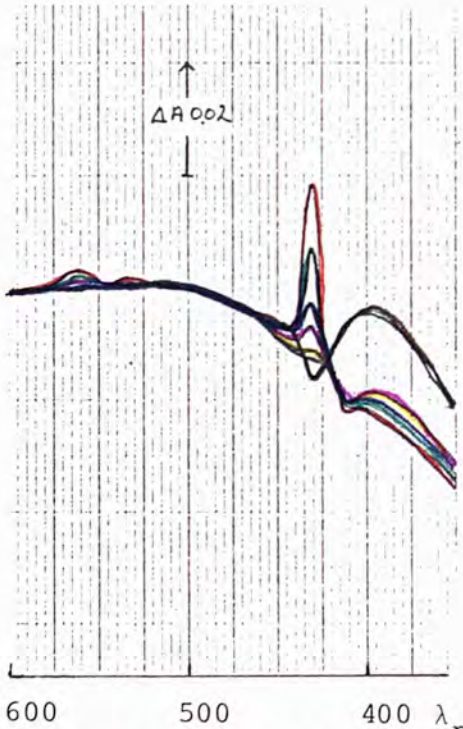


Fig. 2.3.7.b

Further experiments showed that the magnitude and the direction of the response is concentration-dependent.

Fig. 2.3.7.a shows that at • 8, • 4, • 2, • 1, • 0.5 mM ATP (final conc.) there is an increase in the Fe^{3+}/Fe^{2+} ratio of helicorubin, whereas at • 0.25 mM ATP a small decrease is observed.

At the start of the experiment, helicorubin is 72% in the reduced state.

Fig. 2.3.7.b shows that the opposite effect is found with ADP. At • 8, • 4, • 2, • 1 mM ADP (final conc.) there is a decrease in the Fe^{3+}/Fe^{2+} ratio of the haemoprotein, while at lower concentrations, i.e. • 0.5 and • 0.25 mM ADP, an increase in the ratio is observed.

At the start of the experiment, helicorubin is 72% reduced.

No response is obtained with either ATP or ADP at concentrations below 0.25 mM.

The change in the redox state of helicorubin on addition of ATP or ADP was measured by difference spectrophotometry in the usual way. (See 2.2.9.). The reaction was started on addition of 0.1 ml of either nucleotide in 0.1 M Tris-HCl buffer, pH 7.0, at 2.5-80 mM concentration to 0.9 ml of 5.5% egg homogenate in buffer. The change in the redox state was measured after 10 min. incubation at $30^{o}C$.

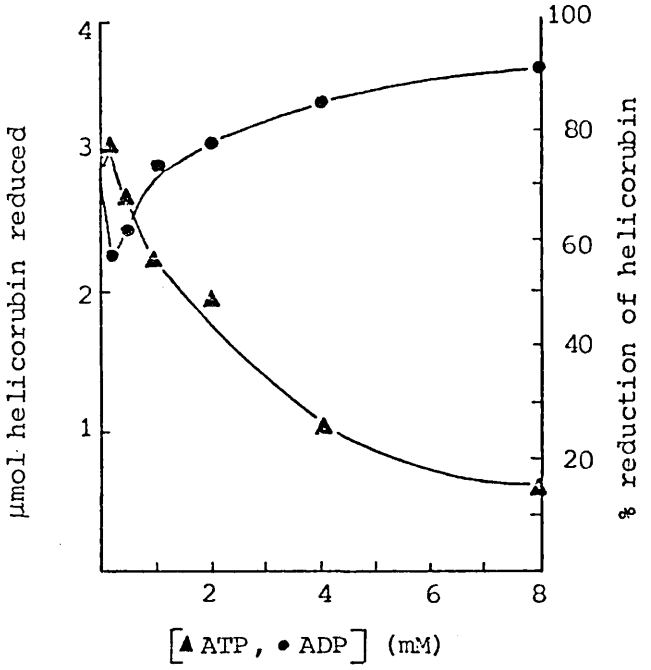

µmol helicorubin reduced
% reduction of helicorubin
[▲ ATP, ● ADP] (mM)
4
3
2
1
100
80
60
40
20
2
4
6
8

Fig. 2.3.8.

Fig. 2.3.8. The change in the degree of reduction of helicorubin on addition of ATP or ADP (final conc. 0.25-8 mM)

The results shown represent the means of four determinations (including that in Fig. 2.3.7.a and b) carried out on each of the two above-mentioned (Table 2.3.4.) independently prepared homogenates. For experimental details see Chapter 2.2.9.

The response to GTP, investigated in a single instance, although quantitatively similar to that obtained with ATP, only amounted to some 20% of that obtained with the latter.

Further experiments revealed a rather more complex situation; on storage of the homogenate in buffer at pH 7.0, perfusion with oxygen, dialysis against water or passage through a Sephadex G-10 column, the Fe^{3+}/Fe^{2+} ratio of helicorubin in the homogenate increased. Increased ratios are also observed with homogenates of partially developed eggs. Results obtained with these show that the change in redox state obtained on addition of ATP or ADP is related to the Fe^{3+}/Fe^{2+} ratio of haemoprotein at the start of the reaction.

From a study of twenty six independently prepared egg homogenates the overall picture summarised in Table 2.3.5. has emerged, suggesting that homogenates may be classified on the basis of their response to adenine nucleotides into four broad categories (A-D).

	Initial percentage reduction of helicorubin P			
Nucleotide added	A 95-85	B 85-70	C 70-50	D 50-20
	Initial effect of nucleotide on the redox state of the haemoprotein			
ATP	Oxidation	Oxidation, sometimes preceded by a transient reduction at low nucleotide conc. (<0.5 mM)	Oxidation at nucleotide conc. above 1mM, reduction at lower conc.	Reduction
ADP	Oxidation	Reduction at conc. of 2 mM and above. At lower conc. reduction may be preceded by a transient oxidation.	Reduction	Reduction

Table 2.3.5.

The relationship between the Fe^{3+}/Fe^{2+} ratio of helicorubin and the effects of nucleotides added to the homogenate. (The experiments were carried out as described in Chapter 2.2.9.)

A: Homogenates where the haemoprotein is 85-95% in the reduced state. Such a high degree of reduction is found in freshly prepared homogenates of eggs collected immediately after laying. Both ATP and ADP invariably effect an initial oxidation of helicorubin. This oxidation represents the opening phase of a slow oscillatory phenomenon, in which helicorubin is alternately oxidised and reduced. The initial response is always greater when ATP is added (10 homogenates of this type were investigated.)

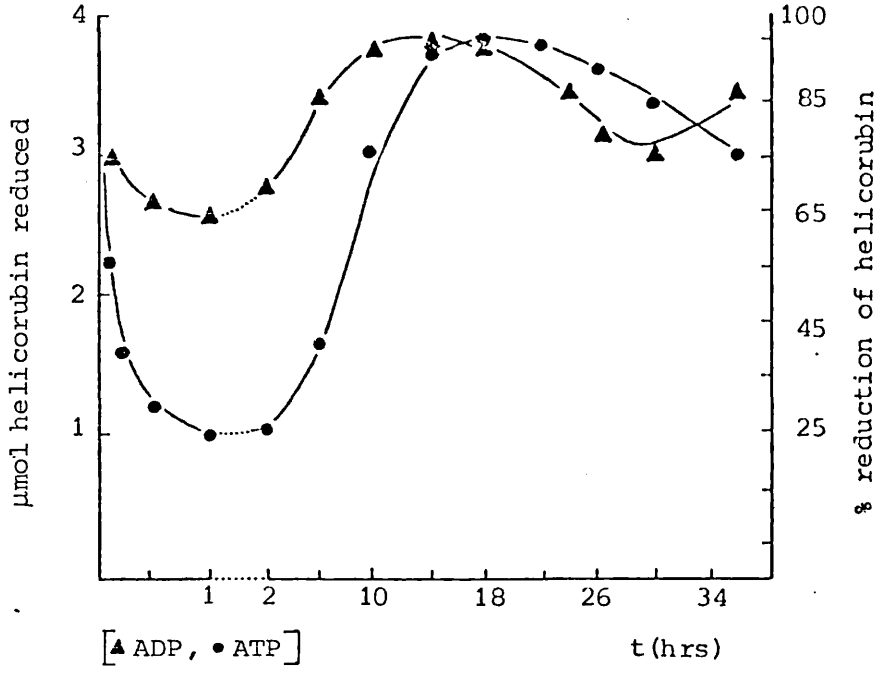

4
3
2
1
100
85
65
45
25
µmol helicorubin reduced
% reduction of helicorubin
1
2
10
18
26
34
[▲ ADP, • ATP]
t(hrs)

Fig. 2.3.9.

Fig. 2.3.9. The change in the reduction of helicorubin after addition of ATP or ADP (10 mM final conc.) to the homogenate observed over 36 hrs

At the beginning of the reaction, the helicorubin was 85% reduced.

The results represent the mean values for experiments carried out in duplicate with two independently prepared homogenates. Similar results were obtained with eight other homogenates where the haemoprotein was between 85 and 95% reduced.

For experimental details, see Chapter 2.2.9.

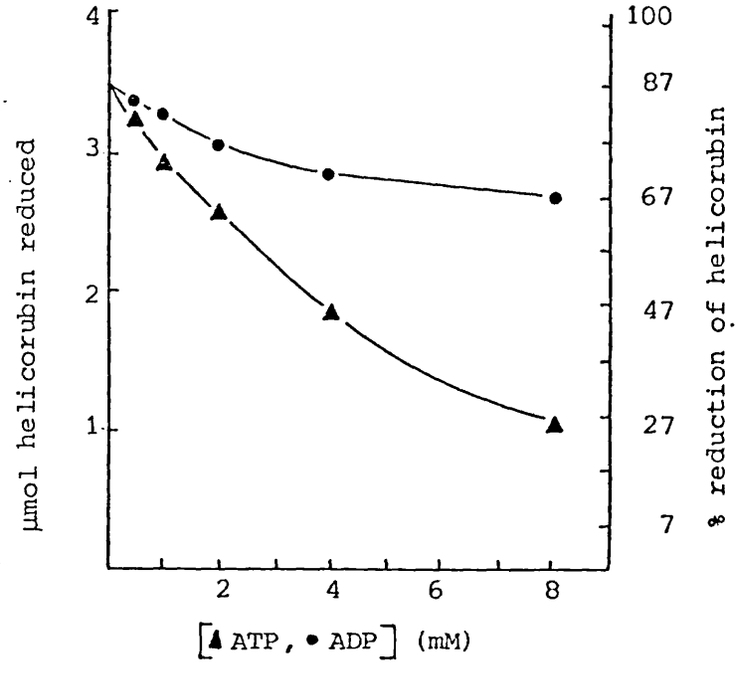

4
3
2
1
100
87
67
47
27
7
2
4
6
8
µmol helicorubin reduced
% reduction of helicorubin
[▲ ATP, • ADP] (mM)

Fig. 2.3.10.

Fig. 2.3.10. The change in reduction of helicorubin (initially 87% reduced) 10 min. after addition of ATP or ADP (1 mM final conc.) to a 5% (w/v) egg homogenate

The results were obtained in duplicate from two independently prepared homogenates. Similar observations were made with eight other homogenates where the reduction of the haemoprotein was between 85 and 95%.

B: This type of response seems characteristic of homogenates where the haemoprotein is between 70 and 85% in the reduced state. ATP increases the Fe^{3+}/Fe^{2+} ratio of the cytochrome, while addition of ATP usually results in a decrease in this ratio. At concentrations below 2 mM, this reductive phase may be preceded by a transient oxidation.

The oscillatory effects for both ATP and ADP are very similar to that shown in Fig. 2.3.9.

Fig. 2.3.8. shows the response to varying concentrations of ATP or ADP characteristic of this type of homogenate.

Seven observations of this type were obtained, mainly with eggs stored in a deep-freeze compartment for up to 18 months.

C: In these homogenates helicorubin is 50-70% reduced. This is usually observed with homogenates aged for 3 to 7 days, or those prepared from partially developed eggs. At concentrations above 1 mM, ATP usually increases the Fe^{3+}/Fe^{2+} ratio of the cytochrome. At lower concentrations, its addition causes a decrease in this ratio. ADP initially causes the transfer of reducing power to the helicorubin, although transient initial oxidations similar to that described for homogenates of the B-type have occasionally been observed.

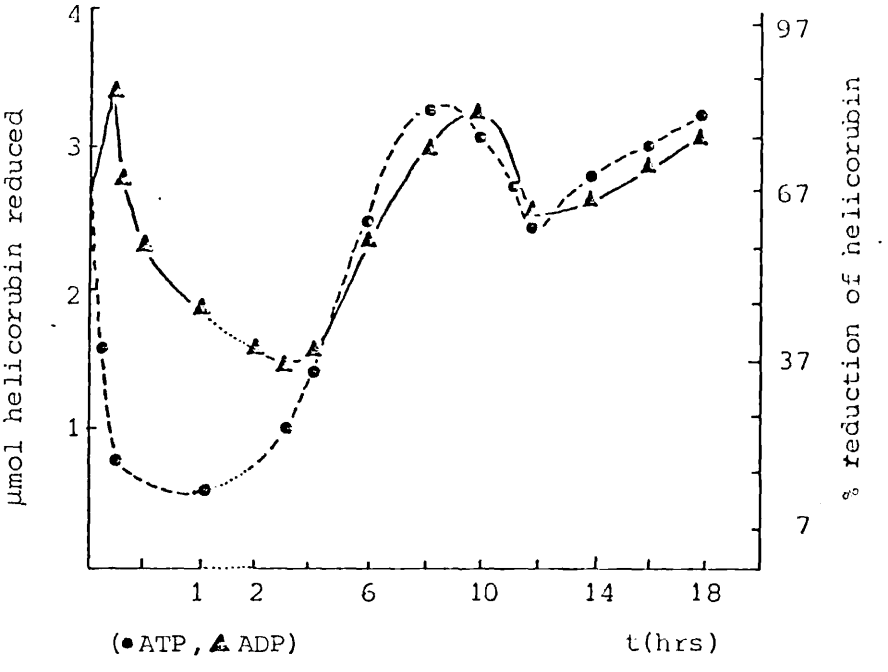


Fig. 2.3.11.

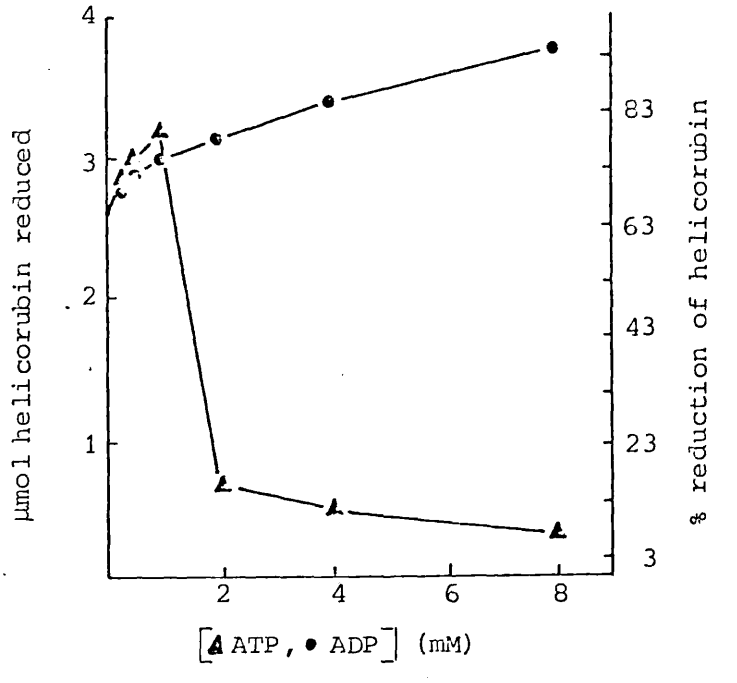


Fig. 2.3.12.

Fig. 2.3.11. The change in the reduction of helicorubin (initially 67% reduced) after addition of ATP or ADP (1 mM final conc.) to a homogenate, observed over a period of 18 hrs

For experimental details see 2.2.9.

Fig. 2.3.12. The effect of adenine nucleotide concentration (1 mM final conc.) on the redox state of helicorubin 10 min. after addition of ATP or ADP to the homogenate. At the start of the reaction, the haemoprotein was 63% reduced.

For experimental details see 2.2.9.

Results shown in Fig. 2.3.11 and 12 represent the mean values of determinations carried out in duplicate on two homogenates. Similar observations were made with five homogenates where the helicorubin was between 50 and 70% reduced.

D: Homogenates where helicorubin is less than 50% reduced. These are usually homogenates investigated 8 to 21 days after preparation or made with eggs on the point of hatching.

Addition of both ATP and ADP lead to a decrease in the Fe^{3+}/Fe^{2+} ratio of helicorubin.

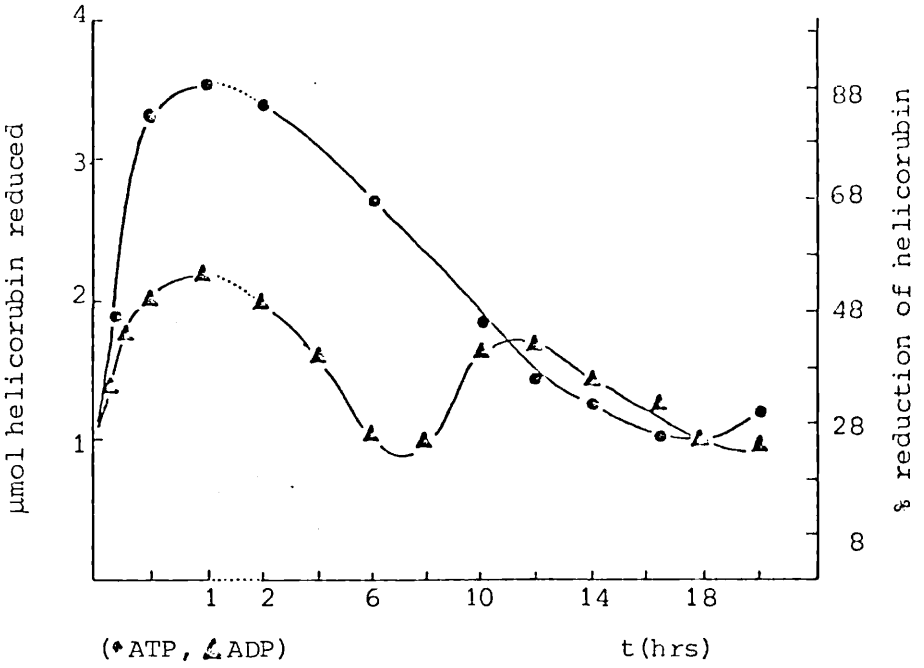


Fig. 2.3.13.

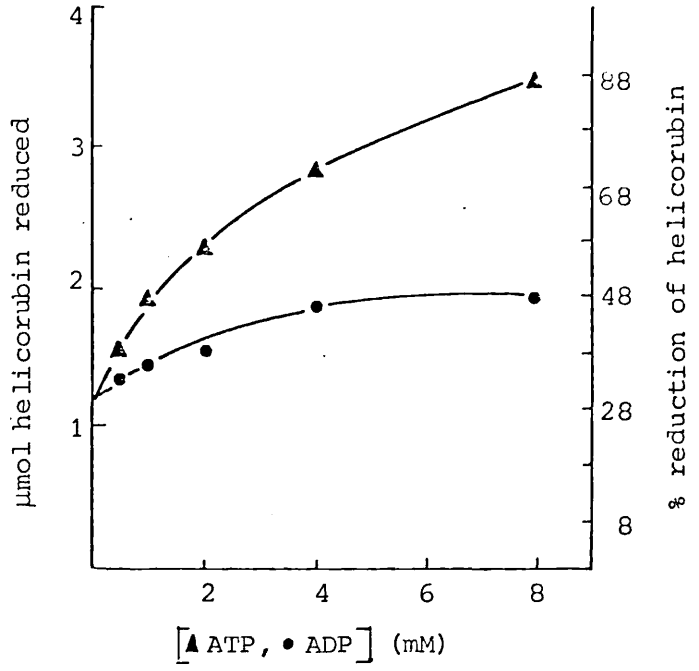


Fig. 2.3.14.

Fig. 2.3.13. The change in the reduction of helicorubin (initially 28% reduced) after addition of ATP or ADP (1 mM final conc.) to a homogenate.

Fig. 2.3.14. The effects of ATP and ADP, 10 min. after addition, on the reduction of helicorubin in an egg homogenate (initial reduction = 28%).

Results shown in Fig. 2.3.13 and 14 represent the mean values from three determinations carried out in triplicate on one homogenate. Similar observations were made with three other homogenates where the degree of reduction of helicorubin was below 50%. (For experimental details see 2.2.9.).

2.3.4. Further evidence for the dependence of the response to adenine nucleotides on the initial redox state of helicorubin

The observations described above showed that the homogenate possesses oxidising and reducing power which can be released by adenine nucleotides. Furthermore, they suggested that the initial degree of reduction of the haemoprotein governed the response to adenine nucleotides.

Further support for this was obtained from studies where a freshly prepared homogenate (Type A) in which the cytochrome was 87% reduced, was either
i) dialysed for 24 hrs at $4^{o}C$ against 10 ℓ distilled water, or
ii)passed through a Sephadex G-10 column.

These processes led to a change in the reduction level of the haemoprotein, presumably because of the removal of low-molecular substrates.

The effects of added ATP and ADP on the untreated homogenate and on those dialysed or passed through a Sephadex column, were investigated.

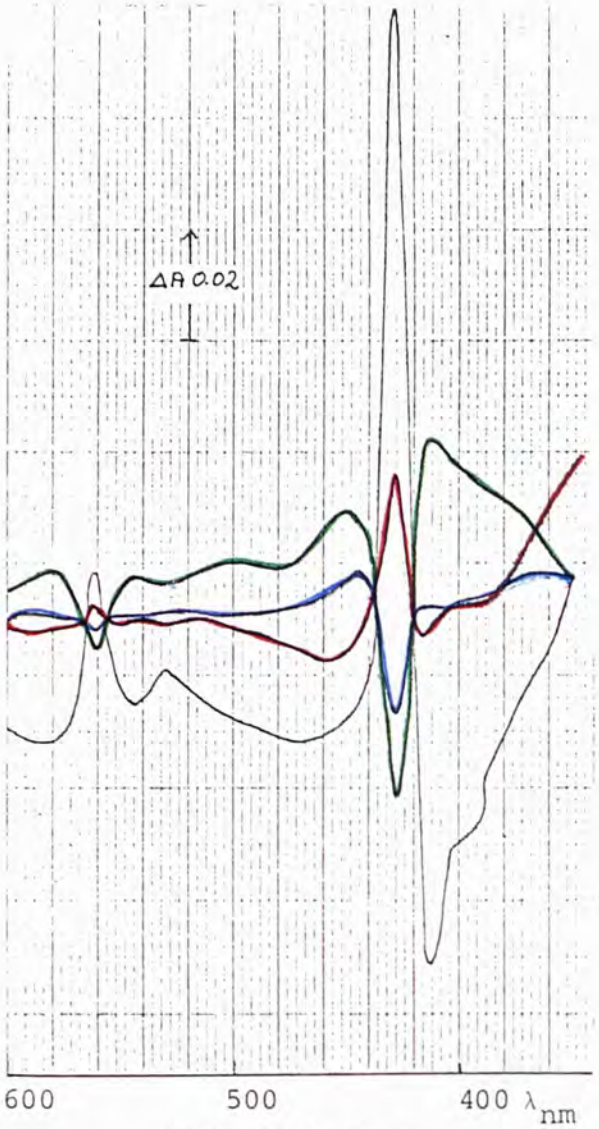


Fig. 2.3.15.c

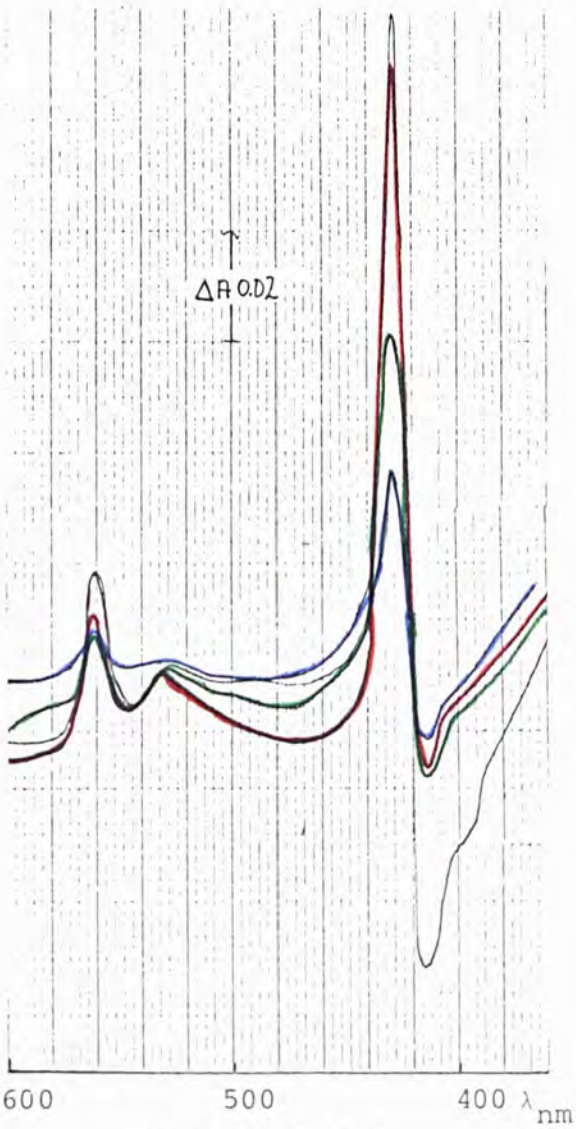


Fig. 2.3.15.b

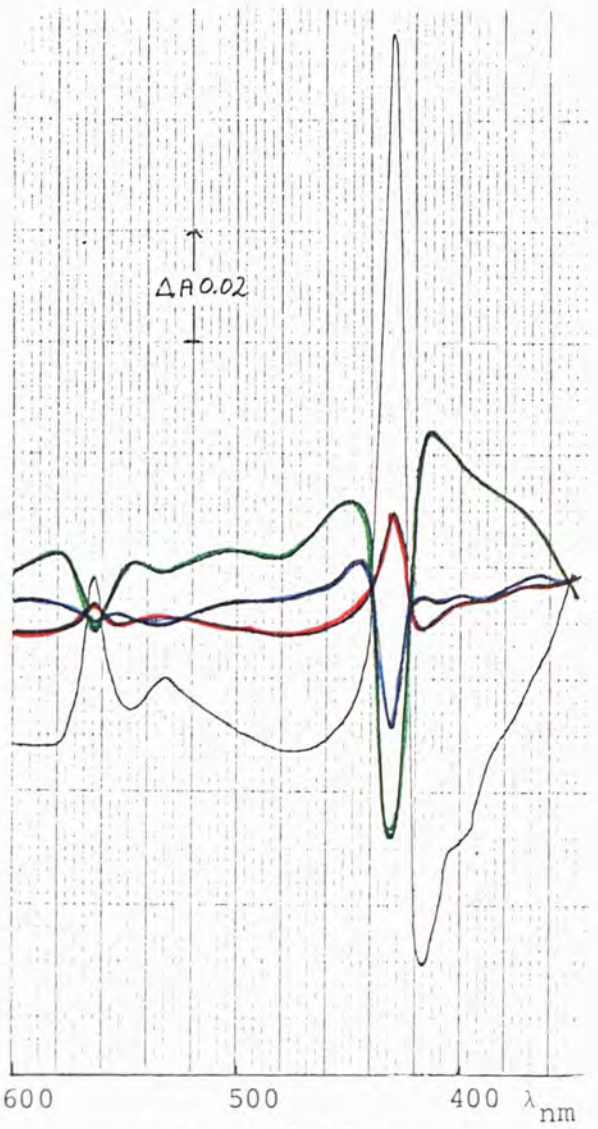


Fig. 2.3.15.a

Fig. 2.3.15.a Response of the untreated homogenate to adenine nucleotides

The change in difference spectrum 10 min. after addition of 1 mM ATP or ADP (final conc.) to the homogenate in the test cell was monitored as described in Chapter 2.2.9.

- reduced homogenate v. oxidised homogenate
- reduced homogenate v. homogenate
- homogenate + ATP v. homogenate
- homogenate + ADP v. homogenate

From these recordings it can be calculated that in the untreated homogenate helicorubin is 87% reduced. 10 min. after addition of ADP this has fallen to 69%, while ATP causes a decrease to 43% in the same time.

Fig. 2.3.15.b Response to adenine nucleotides of the same homogenate after dialysis

For experimental details see 2.3.14.

- reduced v. oxidised homogenate
- reduced v. dialysed homogenate
- homogenate + ATP v. homogenate
- homogenate + ADP v. homogenate

Following dialysis, the haemoprotein was 26% reduced. ADP added to the homogenate raised the reduction level to 53% and ATP to 72%. A Sephadex-treated homogenate gave virtually identical results.

Incubation of either the dialysed homogenate, or that passed through Sephadex, with 50 µM NADH 5 min before the addition of nucleotides, caused an almost complete return to the original degree of reduction and responses to nucleotides similar to those of the untreated homogenate.

Fig. 2.3.15.c The response to adenine nucleotides in the presence of NADH

For experimental details see 2.3.14.

- reduced homogenate v. oxidised homogenate
- reduced homogenate v. homogenate + NADH
- homogenate + ATP v. homogenate
- homogenate + ADP v. homogenate

These observations show that after addition of NADH to the dialysed homogenate a return to almost the original degree of reduction (83%) is obtained. The responses to ADP, i.e. 12% oxidation (18% with the original homogenate) and to ATP, i.e. 37% oxidation (44% with the original homogenate) can thus be considered as similar for both homogenates. These results confirm that the response of a homogenate to adenine nucleotides is dependent on the Fe^{3+}/Fe^{2+} ratio at the start of the reaction.

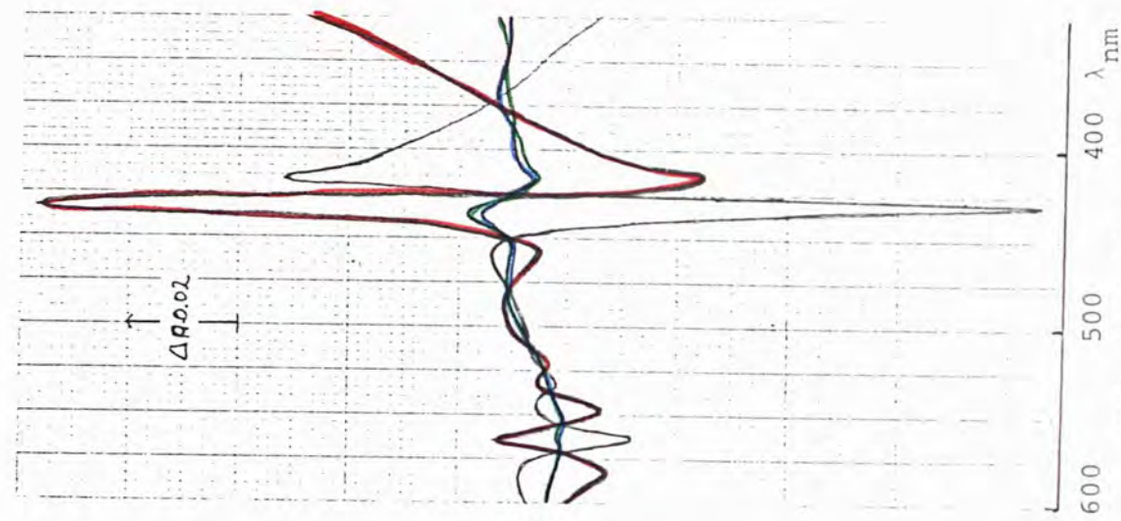


Fig. 2.3.16.a

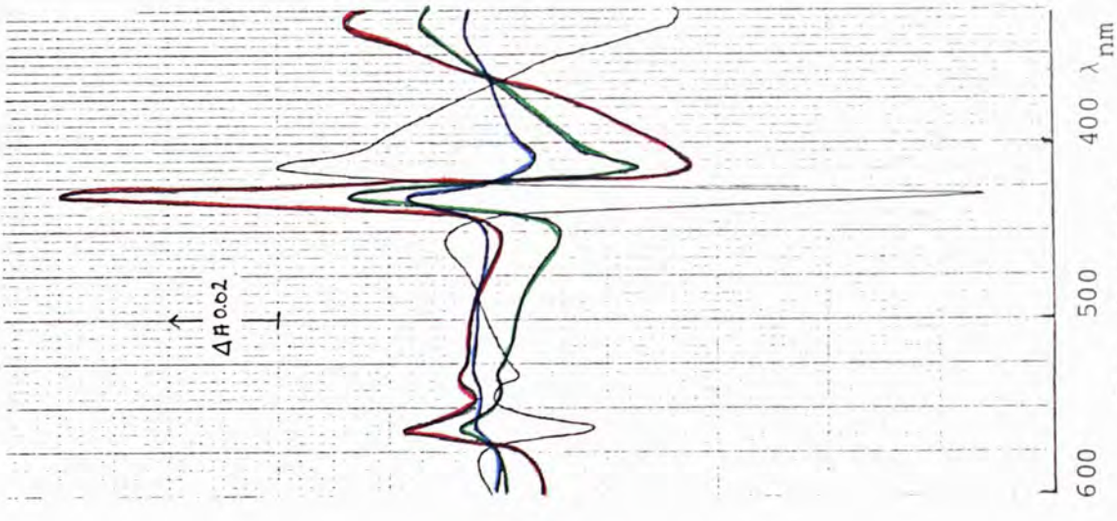


Fig. 2.3.16.b

2.3.5. The influence of ovorubin on the effects of nucleotides on the level of reduction of helicorubin in homogenates

Homogenates in which no ovorubin can be detected spectroscopically after its adsorption on Con A-Sepharose (2.2.2.) show greatly reduced sensitivity to adenine nucleotides. Helicorubin in these homogenates is usually less than 20% reduced.

Fig. 2.3.16.a The effect of ATP and ADP on the reduction level of helicorubin in homogenates freed from ovorubin

The change in difference spectrum 10 min. after the addition of 1 mM ATP or ADP (final conc.) to the homogenate in the test cell was recorded as described in Chapter 2.2.9.

In the preparation shown, helicorubin is 90% oxidised.

- oxidised homogenate v. reduced homogenate
- reduced homogenate v. homogenate
- homogenate + ATP v. homogenate
- homogenate + ADP v. homogeante

Addition of ATP and ADP leads to increases in the reduction of helicorubin by 12 and 8% respectively.

Fig. 2.3.16.b The effect of ATP and ADP on the level of reduction of helicorubin in a reconstituted homogenate

Reconstitution was carried out as described in 2.2.2. The change in difference spectrum on addition of adenine ncueotides was monitored as described in 2.2.9.

- oxidised homogenate v. reduced homogenate
- reduced homogenate v. control homogenate
- homogenate + ATP v. homogenate
- homogenate + ADP v. homogenate

After reconstitution, the haemoprotein is 88% oxidised. Addition of ATP and ADP brings the level of reduction to 52% and 27% respectively.

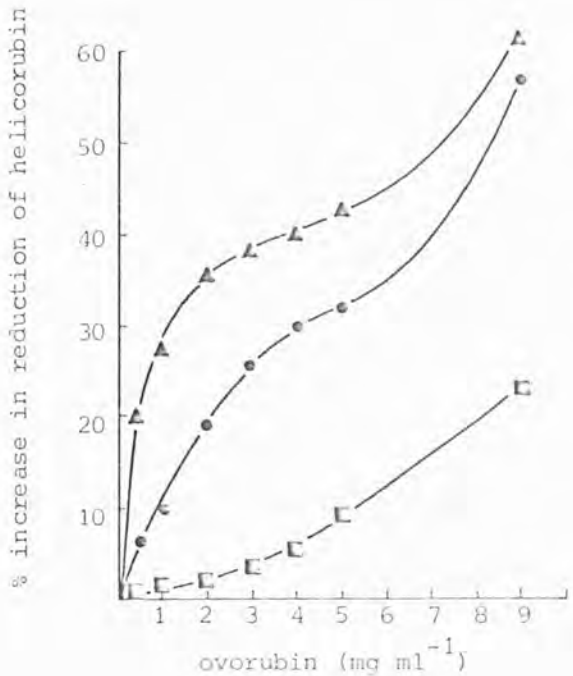


Fig. 2.3.17.a

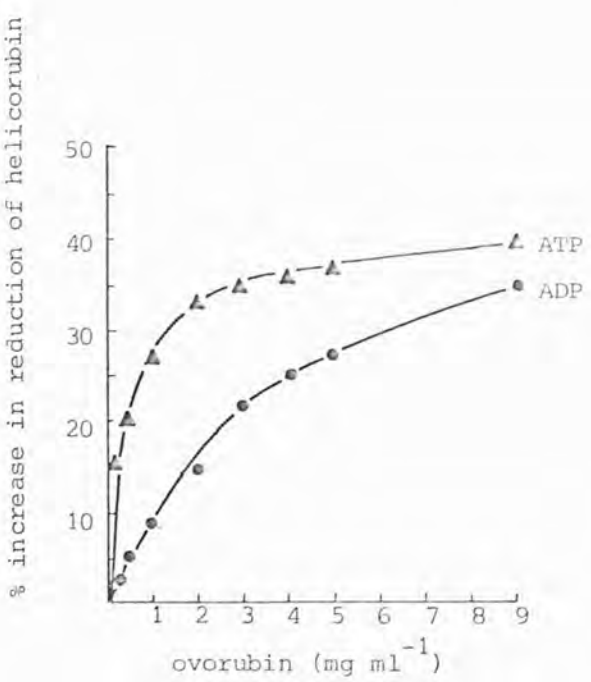


Fig. 2.3.17.b

Observations described above clearly indicate that when ovorubin is returned to homogenates freed from it, the sensitivity to adenine nucleotides is largely restored. Since SDS-PAGE (see Chapter 3) of homogenates treated with Con A-Sepharose still shows residual quantities of ovorubin, it appears likely that total removal of ovorubin would eliminate these effects.

Fig. 2.3.17.a The relationship between ovorubin concentration and the response of helicorubin to adenine nucleotides in a "reconstituted" homogenate

Between 0.25 mg and 9 mg of ovorubin was added to 0.9 ml of a 5.5% (w/v) egg homogenate previously freed from this protein. The change in difference spectrum on addition of 0.1 ml 10 mM ATP or ADP was measured in the usual way after 10 min. incubation at 30°C.

▲ increase in level of reduction of helicorubin after addition of ATP

● the same, after addition of ADP

■ increase in reduction of helicorubin after addition of ovorubin alone.

The relationship between the reduction of helicorubin in the homogenate and the concentration of ovorubin is biphasic in the presence of both ATP and ADP, while in the absence of nucleotides ovorubin reduces the haemoprotein via a reaction of the second or a higher order.

Fig. 2.3.17.b The relationship between ovorubin concentration and the response of helicorubin to adenine nucleotides in a "reconstituted" homogenate corrected for the reduction by ovorubin alone

Experimental details as in Fig. 2.3.17.a.

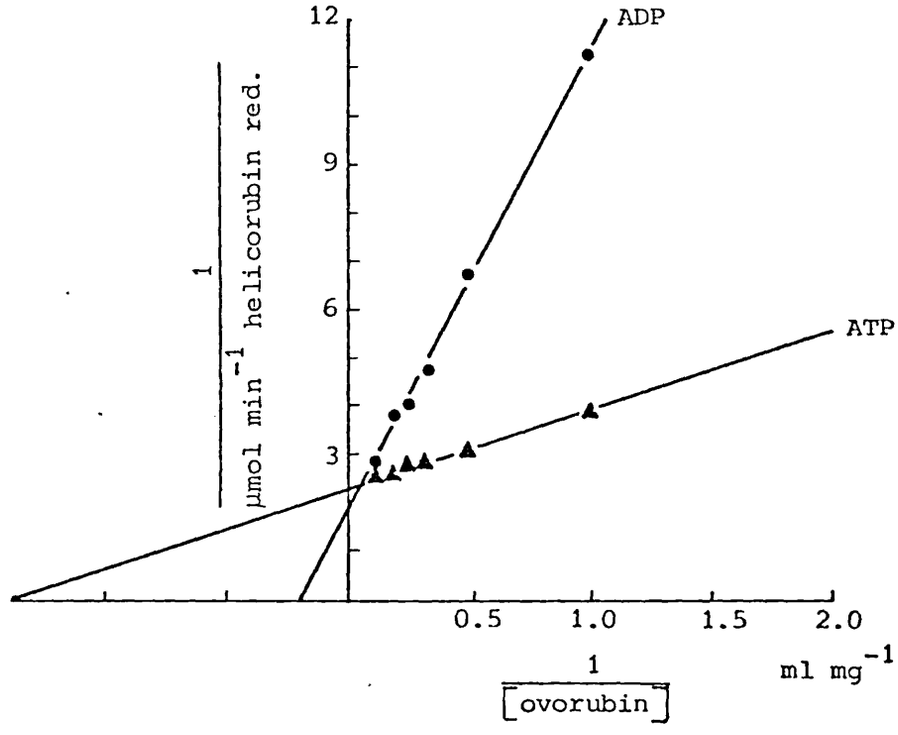

12
9
6
3
ADP
ATP
1
µmol min^{-1} helicorubin red.
0.5
1.0
1.5
2.0
ml mg^{-1}
1
[ovorubin]

Fig. 2.3.18.

Fig. 2.3.18. Lineweaver-Burk plot for the effect of ovorubin concentration on the reduction of helicorubin after addition of ATP or ADP

For experimental details see Fig. 2.3.17.a.

The results shown represent the mean values of four determinations obtained from each of two homogenates.

With the apoprotein of ovorubin, essentially identical results were obtained in a single experiment carried out in duplicate. The apparent K_m for ovorubin in the presence of ADP is 5.55 mg ml^{-1}, V_{max} = 0.22 µmol min^{-1} helicorubin reduced, in the presence of ATP, K_m is 0.55 mg ml^{-1} and V_{max} = 0.185 µmol min^{-1} helicorubin reduced.

No oscillations were obtained with reconstituted homogenates in the presence of either ATP or ADP.

2.3.6. The relatioship between Fe^{3+}/Fe^{2+} ratio of helicorubin and the change in ATP content of the homogenate after addition of ADP + Pi

The response of helicorubin in the homogenate to adenine nucleotides was in some ways reminiscent of the energy-dependent redox reactions of cytochromes of the b_T-type (Flatmark and Pederson, 1973; Wilson *et al.* 1974). Since it is suspected that such a behaviour is connected with participation of the cytochrome in energy transduction at coupling site 2 in mitochondria, the possibility was investigated that energy-linked phosphorylation might occur in egg homogenates from *P. canaliculata*.

Most homogenates used in these experiments corresponded to either type B or C (see Table 2.3.5.). The initial ATP content of the eggs investigated varied between 2.5 and 5.4 nmol ATP g^{-1} wet weight.

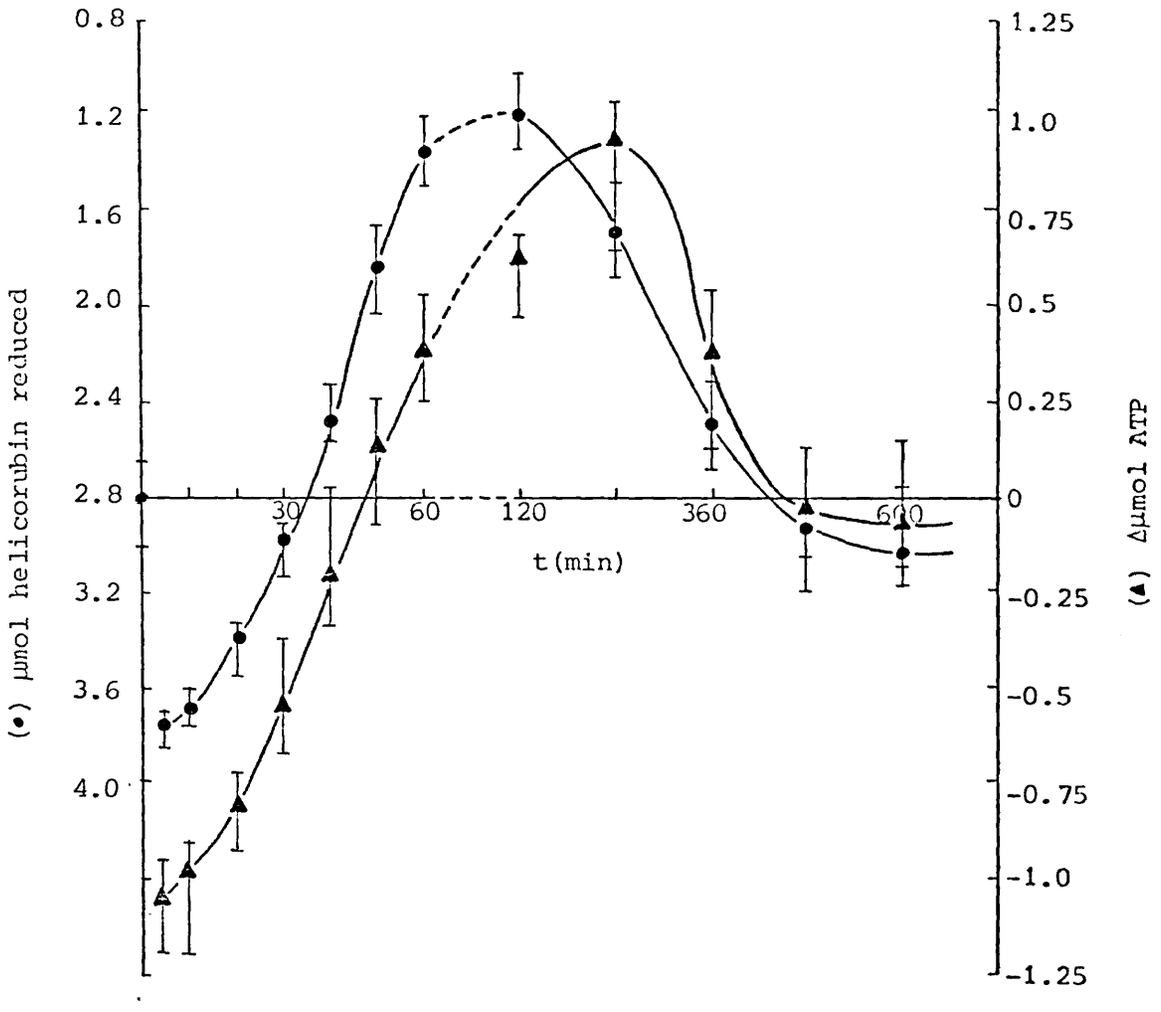

0.8
1.2
1.6
2.0
2.4
2.8
3.2
3.6
4.0
(•) µmol helicorubin reduced
1.25
1.0
0.75
0.5
0.25
0
-0.25
-0.5
-0.75
-1.0
-1.25
(▲) Δµmol ATP
30
60
120
360
600
t(min)

Fig. 2.3.19.

Fig. 2.3.19. The relationship between the redox state of helicorubin and the ATP concentration in a 5% (w/v) egg homogenate (centrifuged for 30 min. at 2,000g, 4°C) after addition of 1 mM ADP + Pi (final conc.) observed over a period of 10 hrs

Changes in the redox state of helicorubin and ATP content were measured as described in 2.3.2. The results shown represent the mean values and the range of three determinations obtained from each of four different homogenates where the initial reduction of helicorubin lay between 63 and 74%.

The reduction of helicorubin on addition of ADP + Pi was invariably associated with a decrease in the ATP content. The molar ratio between ATP broken down and helicorubin reduced is in the range 1.18 to 1.05 (mean 1.08). Since all commercial ADP preparations used in these experiments contained 0.6-3% (w/v) ATP, the latter may provide the energy source for the observed reductive dephosphorylation.

During the succeeding oxidative phase there is an apparent ATP synthesis and 0.46-1.14 μM ATP is synthesised per μM helicorubin oxidised, with the lower ratios found during the later stages of the experiment. The phase shift between the change in the redox state of helicorubin and that in ATP content is a manifestation of this.

Phosphorylation appears to be dependent on the presence of ovorubin, since homogenates freed from the carotenoglycoprotein no longer display ATP synthesis. Return of ovorubin to such homogenates, however, does not restore ATP synthesis.

Although the initial reductive phase was always associated with a decrease in the ATP content of the homogenate, investigations on some eight homogenates showed no ATP synthesis. With homogenates of eggs removed immediately from the animal after issuing from the mantle cavity, the effects of both ATP and ADP + Pi are very much reduced; ATP synthesis has never been observed in such homogenates.

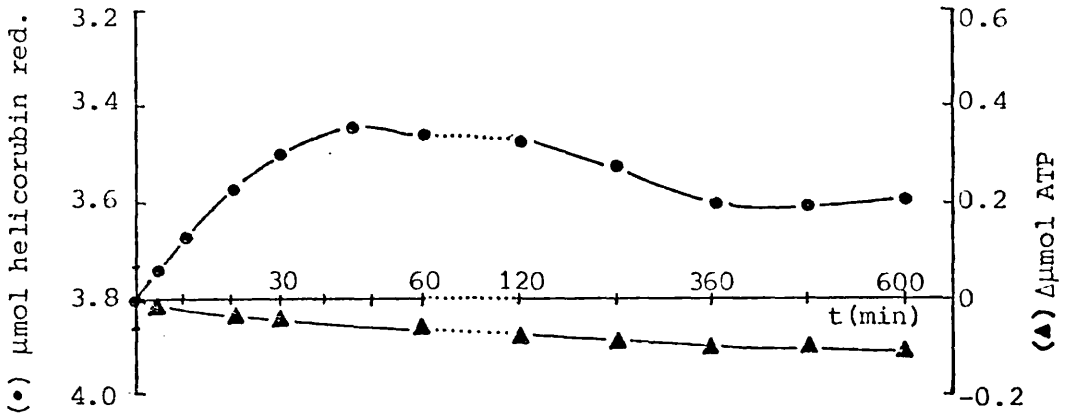


Fig. 2.3.20.

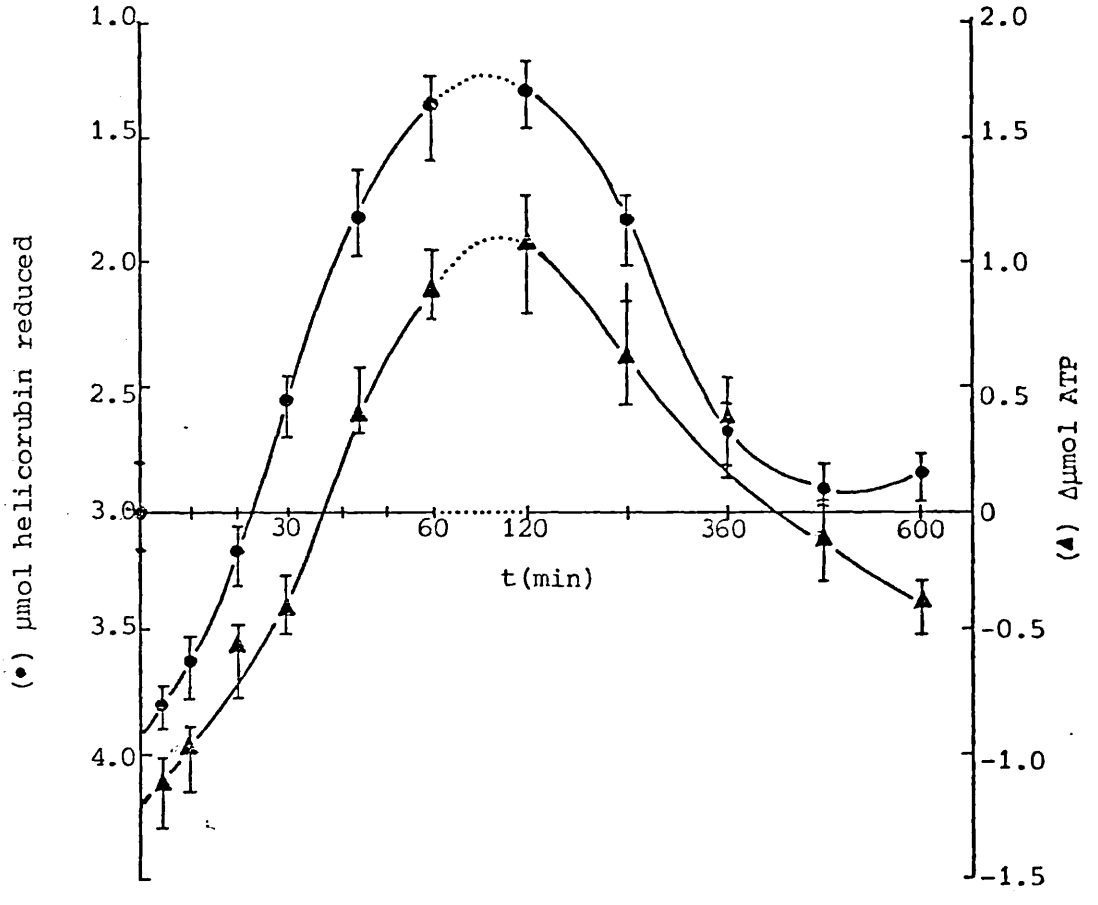


Fig. 2.3.21.

Fig. 2.3.20. The relationship between the redox state of helicorubin and the ATP concentration in a 5% (w/v) egg homogenate (centrifuged for 30 min. at 2,000g) after addition of 1 mM ADP + Pi (final conc.)

This egg homogenate was prepared within 60 min. of issue of the eggs from the mantle cavity of the snail. Helicorubin was 95% in the reduced state. The results shown represent the means of three determinations on a single homogenate.

For experimental detials see Chapter 2.2.11.

It could be shown that the response of helicorubin to adenine nucleotides in the homogenate increased during maturation of the eggs. ATP synthesis on addition of ADP + Pi could only be observed in eggs incubated at 30°C for one hundred hours or more after laying. Addition of 50 μM Ap_5A to homogenates of eggs developed for 100 hrs, to inhibit a potential myokinase activity emerging during egg development, did not affect the results in any way.

Homogenates where ATP synthesis was observed during the oxidation of helicorubin, continue to synthesise ATP after centrifugation for 60 min. at 35,000g in the presence of 10 mM $MgCl_2$.

Fig. 2.3.21. The relationship between the degree of reduction of helicorubin and the change in ATP concentration in a 5% (w/v) egg homogenate (centrifuged at 35,000g for 1 hr, 4°C) in the presence of 10 mM $MgCl_2$ after the addition of 1 mM ADP + Pi (final conc.)

For experimental details see 2.2.11.

The results shown represent the mean values and the range of three determinations made on each of two homogenates. Centrifugation at 85,000g for 60 min. eliminates both the oscillations and the phosphorylation of ADP.

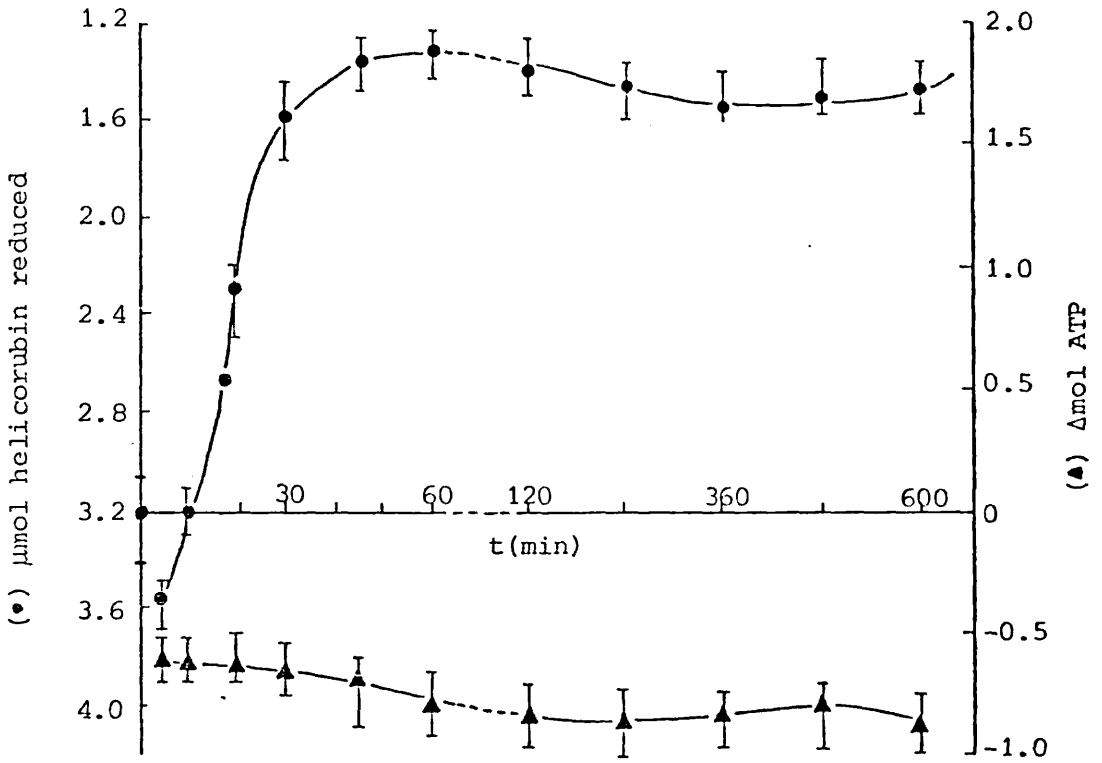

1.2
1.6
2.0
2.4
2.8
3.2
3.6
4.0
2.0
1.5
1.0
0.5
0
-0.5
-1.0
30
60
120
360
600
t(min)
(•) µmol helicorubin reduced
(▲) Δmol ATP

Fig. 2.3.22.

Fig. 2.3.22. The relationship between the state of reduction of helicorubin and the change in ATP concentration in a 5% (w/v) egg homogenate (centrifuged at 85,000g for 1 hr, 4°C) in the presence of 10 mM $MgCl_2$ after the addition of 1 mM ADP + Pi (final conc.

For experimental details see 2.2.11.

2.4. Discussion

2.4.1. Spectral characteristics of the egg homogenate and the effects of O_2, CO and anaerobiosis

The prominent spectral features of the egg homogenate (Fig. 2.3.1a in the visible range are due to the carotenoglycoprotein ovorubin and the haemoprotein helicorubin. In this homogenate, the haemoprotein is 87% in the reduced state, as can be calculated from the difference spectra (Fig. 2.3.1.b). The spectra and difference spectra of the egg homogenate freed from the carotenoprotein show that the haemoprotein is now much more oxidised, i.e. it is ca. 18% in the reduced state. As already mentioned, this decrease in the reduction of helicorubin is much less pronounced when the homogenate is centrifuged at 10,000g than at 2,000g, which is consistent with the presence of a particulate helicorubin oxidase, although no other evidence is found for such a protein in either the egg homogenate or in the homogenate freed of ovorubin. The presence of such an entity is further suggested by studies where the effects on the homogenate of oxygen perfusion and anaerobiosis were examined (Fig. 2.3.3.a).

Oxygenation of the homogenate caused oxidation of helicorubin by some 24%. The observation that anaerobiosis leads to an increase in the reduction of helicorubin is rather reminiscent of the events observed with mitochondria in state 5 (Chance and Williams, 1955), where components of the electron transport chain of lower redox potential than the cytochrome oxidase become progressively more reduced in the presence of substrates under anaerobic conditions. The finding that, on dialysis, the haemoprotein in the egg homogenate also becomes gradually more oxidised (Fig. 2.3.3.b) further suggests that low molecular weight substrates are utilised to reduce helicorubin.

It also seems that the presence of ovorubin is needed for transferring this reducing power to helicorubin since:

1. Removal of ovorubin leads to rapid oxidation of the haemoprotein (Fig. 2.3.2.a and b).
2. Anaerobic conditions do not significantly increase the reduction of helicorubin in homogenates freed of ovorubin,

whereas return of ovorubin leads to a decrease in the Fe^{3+}/Fe^{2+} ratio of the haemoprotein even in dialysed homogenates.

The feeble effect of oxygenation on homogenates freed of ovorubin or on reconstituted homogenates is probably due to the fact that in such preparations helicorubin is largely in the oxidised state.

It is interesting to note in this respect the effect of oxygenation on the spectrum of ovorubin in the carotenoid region where an increase in absorbance can be seen (Fig. 2.3.4). This is in contrast to observations made by Karnaukhov (1971), who claims that there is a reversible decrease in the absorption maxima of carotenoids present in cytosomes of *Lymnea stagnalis* on binding of O_2. It must at present be left undecided whether this author's observation should be taken to indicate a physiologically significant interaction of the carotenoid with oxygen. Difference spectra between ovorubin and a solution of the carotenoprotein perfused with oxygen show spectral features that are consistent with a straight increase in absorbance, while exposure to carbon monoxide (Fig. 2.3.5.a) leads to a hypsochromic shift in the spectrum of the carotenoid.

As can be seen from Fig. 2.3.5.a, exposure of the homogenate to CO leads to a rapid oxidation of the haemoprotein. After dissociation of the gas, helicorubin becomes again gradually more reduced.

2.4.2. The effect of electron transport inhibitors on the redox state of helicorubin in the presence and absence of substrates

Azide and cyanide affect the level of reduction of helicorubin in the homogenate in a very similar fashion to CO in so far as, in the absence of added reducing power, they lead to oxidation of the haemoprotein, while amytal, rotenone, antimycin and n-heptylquinoline oxide produce the opposite effect, but much less pronounced.

There was no significant effect when these inhibitors were added to the homogenate freed from ovorubin. If one were to apply criteria employed in interpreting the effects of inhibitors

on mitochondrial electron transport, these observations could be taken as evidence that helicorubin mediates between two redox functions of ovorubin provisionally called X and Y.

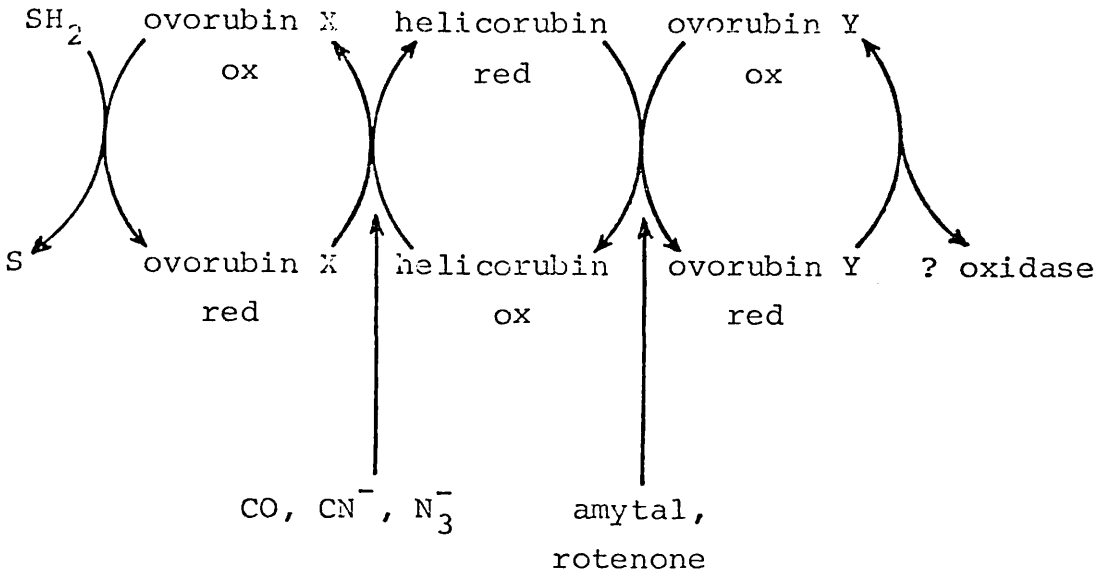


Fig. 2.4.1.

Hypothetical position of helicorubin in an electron transport chain based on the effects of inhibitors on the homogenate.

The effects observed with CN^-, N_3^- and CO, which are classical inhibitors of the terminal oxidase in mitochondria because they inhibit the enzymic transport of electrons to oxygen, suggest that in this particular electron transport system the redox function in ovorubin inhibited by these poisons does not perform the function of a terminal oxidase. It indicates, however, the involvement of oxygen at this site, since O_2, CO, N_3^- and CN^- commonly compete for identical binding sites. The decrease in the Fe^{3+}/Fe^{2+} ratio of helicorubin brought about by amytal, rotenone, n-heptylquinoline oxide and antimycin suggest the presence of one or even two further redox functions of ovorubin with a more positive redox potential than helicorubin.

Fig. 2.3.6. shows that helicorubin in the egg homogenate is reduced by ascorbate, GSH, NADH and NADPH. The latter two substrates have identical V_{max}, so that the same catalytic site may be involved in both cases. The reduction of helicorubin

by these substrates in homogenates freed from ovorubin is greatly diminished. Evidence is presented in Chapter 5 that ovorubin catalyses the reduction of helicorubin and cytochrome *c* from horse heart or *Candida krusei* in the presence of the same substrates. A comparison of the results shows that V_{max} for these substrates is about the same for the homogenate and the isolated proteins and so is the K_m with ascorbate and GSH. The latter constant is greater for NADH and NADPH in the reaction catalysed by the homogenate.

The qualitative effects of inhibitors on the reduction of helicorubin in the egg homogenate and on the reduction of cytochrome *c* by ovorubin in the presence of the above-mentioned substrates are very similar. However, antimycin and n-heptylquinoline oxide are virtually without effect on the latter reaction. The inhibitions by amytal and rotenone are more pronounced on the reduction of cytochrome *c* by ovorubin.

In both systems N_3^- and CN^- increase the reduction of the haemoprotein in the presence of NAD(P)H; in the homogenate there is also a substantial increase in the reduction of helicorubin by ascorbate and GSH in the presence of KCN, which is not observed for the reduction of cytochrome *c* by ovorubin (see Chapter 5.).

The effects observed with the inhibitors in the presence of exogenous substrates appear to be consistent with observations on mitochondrial electron transfer, i.e. inhibition of electron transfer from substrates by amytal, rotenone, antimycin and n-heptylquinoline oxide by interaction of the metabolic poison with a flavin or non-haem iron prosthetic group and the inhibition of a cytochrome oxidase by CO, N_3^- and CN^-.

The findings leading to the above interpretations are, however, difficult to reconcile with the earlier mentioned effects of these inhibitors on the homogenate in the absence of exogenous substrates, which suggest an opposite arrangement of electron carriers (Fig. 2.4.). From comparisons with known electron transport systems, no analogy can be derived. Comments on the mechanism of inhibition are purely speculative.

The effects of inhibitors on the reduction of cytochrome *c* by ovorubin in the presence of substrates are discussed in detail in Chapter 5.4., where it is suggested that the enhancement by N_3^- and CN^- of the reduction of cytochrome *c* in the presence of NAD(P)H may be due to a number of factors. These include the inhibition of a potential terminal oxidase function in ovorubin or the formation of cyanide adducts to the nicotinamide rings which favour the dissociation of a proton adjacent to the highly polarised C ≡ N adduct. It is conceivable that these explanations also hold for the egg homogenate. The proposal does not, however, explain the activation by CN^- of the reduction of the haemoprotein by ascorbate and GSH, which is not observed with the isolated protein.

The third explanation proposed in Chapter 5 is based on the observation that $O_2^{\cdot -}$ generated by ovorubin reduces the haemoprotein, a reaction which is inhibited by superoxide dismutase (SOD) (McCord and Fridovich, 1968, 1969). Commercial preparations of cytochrome *c* usually contain trace amounts of SOD, which is inhibited by CN^-, thus leading to an increase in reduction of the haemoprotein. It appears, however, improbable that the inhibition of an endogenous SOD by CN^- is responsible for the observed enhancement of the reduction of helicorubin in the presence of added substrates, since the same, and not the opposite, effect should be obtained in their absence. It will be necessary, however, to establish whether or not SOD is present in the eggs, in view of the finding that $O_2^{\cdot -}$ is produced by ovorubin. This radical could give rise to such cytotoxins as $OH^{\cdot}$ radicals or singlet oxygen (see Chapter 1.). If SOD could not be found, this would point to a function of the carotenoid moiety of ovorubin in protecting the eggs from harmful intermediates produced in univalent reduction of molecular oxygen by the same protein.

At this point, it appears worthwhile to mention another electron transport system on which CO, azide and cyanide have effects. This is the nitrogenase system which catalyses the six-electron reduction of N_2 to ammonia. The e.s.r. spectrum of this protein shows signals at g = 1.94 and 4.2. Similar signals

are obtained with ovorubin. This nitrogenase enzyme system is also able to reduce other compounds,

e.g. $N_3^- + 4H^+ + 2e^- \longrightarrow N_2 + NH_4^+$

$CN^- + 8H^+ + 6e^- \longrightarrow CH_4 + NH_4^+$

CO is not reduced by nitrogenase but is a potent non-competitive inhibitor. O_2 rapidly inactivates nitrogenase.

Although there is no evidence that an analogous function is associated with ovorubin, a similar pathway might be followed in which ovorubin could pass electrons to CN^- and N_3^- in preference to helicorubin.

However, there is no obvious reason why this should not be observed in the presence of excess substrate.

Although these inhibitor studies failed to yield conclusive information concerning the nature or the sequence of the components of the electron transport chain, they gave evidence for the interaction of ovorubin and helicorubin in such a system. They also suggested that more than one redox function is associated with ovorubin and takes part in the electron transport.

2.4.3. The effect of adenine nucleotides on the degree of reduction of helicorubin in the homogenate

Experimental evidence obtained up to this point clearly showed the presence of an electron transport arrangement involving ovorubin and helicorubin.

Results by Keilin (1957) showed that helicorubin from *Helix* had E_o = 0.2 V, a value occupying an intermediate position between those of cytochrome *b* and cytochrome *c*.

Studies on mitochondria showed that cytochromes of the *b* type undergo a change in redox state on energisation of the mitochondria by ATP (Flatmark and Pederson, 1973; Wilson *et al*, 1974a, b). This led to the proposal that *b*-cytochromes, in particular b_T , play a direct role in mitochondrial energy transduction. It was therefore decided to look for similar effects following addition of adenine nucleotide to the homogenate. The results clearly suggested the presence of a nucleotide-modulated electron transport system in the egg

homogenate. The type of response to adenine nucleotides is dependent on the initial degree of reduction of helicorubin and on the nucleotide concentration.

In mitochondria, the E_m values of three components, i.e. cytochrome a_3 at site 3, cytochrome b_T and c_1 at site 2, and the iron-sulphur centres at site 1, are changed on addition of ATP, which according to Wilson *et al*, (1974) suggests the existence of a coupling between their chemistry and the phosphorylation state ratio.

The E_m of cytochrome b_T increases in the presence of high concentrations of ATP; this is taken as evidence that an energised oxidised form is involved in energy transduction. Furthermore, a fall in the value for cytochrome a_3 by some 0.230 V in the presence of ATP, led to the proposal that a high-energy reduced form of a_3 is coupled to the synthesis of ATP.

Such conclusions obviously depend greatly upon both the precision and the activity with which the redox ratios and the phosphorylation state ratio, R_p, can be measured. ($R_p = \frac{[ATP]}{[ADP][Pi]}$)

Unfortunately experiments described so far do not allow for any quantitative conclusions, since the commercially obtained preparations of ATP and ADP were impure (Chapter 4.), the degree of impurity varying from preparation to preparation to an extent that was only appreciated during the later stages of the investigation.

The effects elicited by ATP and ADP are influenced by various inhibitors. The most striking effect is obtained with KCN, which inhibits the oxidation of helicorubin promoted by ATP and changes the reduction observed in the presence of ATP to oxidation.

The inhibition by ATP of the effect of KCN on the mitochondrial cytochrome oxidase has been extensively studied by Wilson and Fairs (1974). They suggest that ATP favours a molecular interconversion of the haemoprotein to a form that binds CN^- with a much lower affinity. It appears conceivable that a similar mechanism may hold in this case, since both ATP and KCN added singly cause stronger oxidation than when they are added

together. ADP has less power than ATP to overcome the effect of KCN, even though its effect on the homogenate alone is opposed to that of KCN.

In all instances observed, the initial change in the redox state of helicorubin brought about by adenine nucleotide is the opening phase of an oscillatory effect in which the haemoprotein becomes alternately more oxidised or reduced, although this is heavily damped after the first cycle.

In mitochondria, periodic variations of the redox state of respiratory carriers can be recorded (Hess and Boiteux, 1971, Fig. 3.). Although it could be shown that all variables analysed oscillated with the same frequency, distinct phase shifts could be observed. The shift of a whole set of variables on addition of ADP or ATP to substrate-fed mitochondria is thought to demonstrate control functions of the energy-coupling mechanism. Cyclic changes of mitochondrial respiration have a period of ca. 30 min. and are thus much faster than those observed with the degree of reduction of the haemoprotein in the egg homogenates.

The observation that removal of ovorubin from homogenates greatly reduces the sensitivity of the haemoprotein to adenine nucleotides, while its return fully restores these reactions, clearly establishes the participation of ovorubin in a nucleotide-modulated electron transport chain. Fig. 2.3.17.a and b, which show the relationship between ovorubin concentration and the response to adenine nucleotides in a reconstituted homogenate, suggest that ovorubin acts as a co-substrate, also catalysing the release of reducing power from the homogenate, since classical Michaelis-Menten kinetics are observed. Initial observation had suggested that the reduction of helicorubin in the homogenate, with increasing concentration of ovorubin was biphasic.

Controls showed that ovorubin reduces the haemoprotein in the absence of added reducing power. This was subsequently followed up in Chapter 5 where it is shown that ovorubin can reduce helicorubin and cytochrome *c* in the presence and absence of reducible substrates.

No oscillations were observed with "reconstituted" homogenates, indicating that some factor is lost during removal of ovorubin from the homogenate and its purification on either CaP gel or Con A-Sepharose.

2.4.4. The relationship between the degree of reduction of helicorubin and the change in ATP concentration of the homogenate after addition of ADP + Pi

The effect of both ATP and ADP on the degree of reduction of helicorubin in the egg homogenate, suggested a similarity of helicorubin with cytochromes of the b_T-type, which are thought to be involved in energy transduction. The possibility that ATP synthesis from ADP and Pi might be associated with these events was therefore investigated. The experiments were carried out under conditions identical with those employed for measurements in the change of reduction of helicorubin. It is unlikely that any increase in the level of ATP observed is due to oxidative phosphorylation in mitochondria from the developing embryo, which would probably not survive under such ionic conditions.

Preliminary investigations were carried out on eggs of uncertain stages of development collected in Paraguay. With homogenates of some of these, synthesis of ATP was observed on addition of ADP and Pi, and there appeared to be a correlation between the state of reduction of helicorubin and the ATP level of the homogenate. The increase in reduction of the cytochrome was always associated with a stoichiometric decrease in the level of ATP, an event that appears to be comparable with reductive dephosphorylation observed in mitochondria (Flatmark and Pederson, 1973). Since the endogenous ATP level in the homogenate is too low to achieve the observed degree of reduction, it is suggested that ATP contained in the preparation of ADP provides the energy.

In homogenates where ATP synthesis was observed, the P/2e ratio varied between 0.92 and 2.28. The higher ratios were commonly found during the early stages of the experiment. These results are rather surprising, since even when one-electron carriers such as cytochromes are involved, the passage of two electrons is required to synthesize one molecule of ATP.

The possibility can therefore not be excluded that additional coupled electron transfer occurs under these conditions. This apparent oxidative phosphorylation and reductive dephosphorylation persists in the supernatants of homogenates centrifuged at 35,000g for one hour. This rules out the participation of particles of mitochondrial or microsomal size. Since no phosphorylation associated with electron transport has been observed in the absence of membrane-dependent translocation of hydrogen ions, the key to this question might be found in the tendency of ovorubin to associate into polymers (see Chapter 3.), possibily leading to vesicle formation in aqueous solution.

The phosphorylation seems to be dependent on the presence of ovorubin, although the return of this protein to homogenates from which it has been removed, does not restore their phosphorylating power. The reason for this apparently irreversible loss of coupled phosphorylation may be twofold:

1. A coupling factor may be removed together with ovorubin from the homogenate.
2. A special spatial arrangement is required for the process. This is disrupted on removal of ovorubin. On the return of ovorubin, the steric confirmation is not regained because the time required for this is longer than the 10 min. preincubation time employed after reconstitution of the homogenate.

Indirect evidence such as the loss of oscillation or the decrease in the phosphatase activity of reconstituted homogenates (Chapter 4.) indicate that treatment of the homogenate with Con A-Sepharose leads to the removal of substances other than ovorubin from the homogenate, which are not recovered after elution of the lectin with α-methylglucose.

Centrifugation at 85,000g for 1 hour eliminates both coupled phosphorylation and the oscillation, providing further evidence that entities other than ovorubin and helicorubin are involved in the process.

Most homogenates investigated showed no ATP synthesis although spectral effects on addition of ADP + Pi were comparable with those that did. While in mitochondria such a situation would

be attributed to uncoupling conditions, this is apparently not the case with the egg homogenates.

In experiments where egg development was monitored, it could be shown that no synthesis of ATP on addition of ADP was observed which could be related to spectral changes in the haemoprotein until ca. 100 hrs after laying. The possibility must therefore be considered that there is an alteration of the electron transport system by a substance secreted from the ovum, or after its incorporation into the embryo. Qualitative considerations rule out that ovorubin and helicorubin are part of the ovum. According to Hylton Scott (1934), in freshly laid eggs, which are 2-3 mm in diameter and surrounded by a calcareous shell, the diameter of the ovum is ca. 50 µm. It is suspended in an apparently undifferentiated egg jelly, the mass of which exceeds that of the ovum by a factor of 1.25×10^5, and the ovorubin content of an egg is at least 5×10^3 the weight of the ovum.

This suggests that in the egg jelly of a mollusc, which has commonly been regarded as an inert and undifferentiated fluid, serving as a nutrient medium for the embryo, ovorubin and helicorubin form part of a nucleotide-modulated electron transport system.

The red colour due to ovorubin appears to be progressively removed from the egg jelly to the embryo during development. At the time of hatching, at least 60% of the ovorubin originally present has been incorporated in the digestive gland.

Whether this involves breakdown and resynthesis in the developing embryo is at present unknown. If an electron transport complex were, in fact, found to be incorporated into the embryo during development, new perspectives would be opened in chemical embryology, since the embryo would have acquired a pre-formed enzyme system of maternal origin.

CHAPTER 3

PURIFICATION AND PROPERTIES OF OVORUBIN AND HELICORUBIN P FROM HOMOGENATES OF THE EGGS OF *POMACEA CANALICULATA*

3.1. Introduction

Ovorubin was first purified by Cheesman (1958) by adsorption on aluminium hydroxide gel and subsequent elution with Na_2HPO_4. Preparations of ovorubin with $E_{280/510} = 2.6\text{-}2.8$ were considered to represent the pure protein and a minimum molecular weight of 335,000 ± 11,000 was reported (Cheesman 1958).

The apoprotein is a glycoprotein; analysis by Norden showed that the carbohydrate component accounts for some 20% of the molecular weight. The presence of glucose and/or mannose end group(s) is suggested because of its interactions with Jack bean lectin concanavalin A (Con A) (Uhlenbruck *et al*, 1976). Adsorption on Con A-Sepharose and subsequent elution of ovorubin with either α-methylglucoside or α-methylmannoside now appears to be a favourable method for purification of ovorubin from freshly laid eggs, since it leads to specific quantitative adsorption and thus production of an ovorubin-free homogenate. In partially developed eggs, however, there is interference from other Con A-reacting glycoproteins. When ovorubin is purified from such eggs, Con A adsorption is therefore preceded by adsorption on calcium phosphate gel from which it can be removed with a solution of 0.2 M Na_2HPO_4 (Cheesman, personal communication).

Cheesman attributed the peak at 280 nm mainly to aromatic amino acids in the protein component, the inflexions at 330 and 480 nm, and the maxima at 510 and 545 to the carotenoid. On the basis of a molar extinction coefficient of 115,000 for astaxanthin (Kuhn *et al*, 1939), he suggested that ovorubin contained one mole of bound carotenoid. He also observed that exposure to daylight and ageing led to a disappearance of the inflexion at 330 nm, while the bands in the visible spectrum underwent minor hypsochromic displacements and changes in relative intensity (Cheesman, 1958, Fig. 4). He suggested that these changes coincided with an early stage in the denaturation of the protein.

The isolation and properties of the haemoprotein helicorubin from the intestinal tract fluid and digestive gland of the land snail *Helix pomatia* were described by Keilin in a series

of papers (Keilin 1956, 1957, 1968; Keilin and Orleans, 1969). Purification was achieved by precipitation of impurities with lead acetate, followed either by paper electrophoresis, or by fractionation on Sephadex G75 and adsorption chromatography on DEAE cellulose (DE32). A value for $E_{276/416}$ of 0.27 is associated with the pure protein.

It is an acidic protein with an isoelectric point in the region of pH 4.3. It is autoxidisable below pH 6 and does not react with CO in neutral or alkaline solutions. It contains no methionine, little lysine and much proline.

3.2. Methods and materials

Chemicals and solvents were of analytical grade where available. Other solvents were redistilled.

Concancavalin A-Sepharose, Sephadex G75 and G200, as well as polyacrylamide gradient gels PAA 4/30 and PAA 21/16, were supplied by Pharmacia, Uppsala, Sweden.

The tracker dyes Pyronin Y and bromophenol blue were obtained from British Drug Houses (BDH), methylene green from Raymond A. Lamb. All other chemicals were supplied by BDH with the following exceptions:
α-methylglucopyranoside and α-methylmannopyranoside by Sigma Chemical Co., bovine serum albumin by Koch-Light, catalase by Worthington. Ceruloplasmin (Type III human), apoferritin (horse spleen) thyroglobulin (bovine) were all obtained from Sigma Chemical Company.

3.2.1. Purification of ovorubin

The eggs from *P. canaliculata* were collected within 3-24 hrs of laying and stored at -20°C for up to 18 months.

For purification of ovorubin and helicorubin, the eggs were removed from the freezer, allowed to thaw at 4°C, homogenised in 0.1 M acetic acid-Na acetate buffer, pH 6.0, containing 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 1 M NaCl with a Potter-Elvehjem homogenizer and subsequently absorbed onto Con A-Sepharose. Adsorption was carried out either as chromatography or as adsorption on the gel followed by removal of the non-absorbing components by vacuum filtration. Before elution, the gel was washed with ten times its volume of acetate buffer to

remove adhering impurities, especially helicorubin. The non-adsorbing materials and the first wash were collected, freeze-dried and used as starting material for the purification of helicorubin. Ovorubin was eluted with either 0.3 M α-methylglucopyranoside or 0.3 M α-methylmannopyranoside, followed by elution with 0.05 M sodium borate buffer, pH 6.0, to remove the more strongly held fraction of ovorubin.

For the purification of ovorubin from partially developed eggs, ovorubin was purified by the method of Cheesman (unpublished observations) which involved its adsorption from the egg homogenate on calcium phosphate gel prepared by the method of Keilin and Hartree (1938). The non-adsorbed materials were removed by vacuum filtration, and the gel was washed with ten times its volume of ice-cold M sodium chloride before elution of ovorubin with 0.2 M Na_2HPO_4. The eluted protein was further purified by readsorption on Con A-Sepharose.

The temperature was kept at $4^{o}C$ throughout the separation.

The apoprotein of ovorubin was prepared by the method of Cheesman (1958).

3.2.2. Protein estimations

Protein estimations were carried out by a modified Folin-Ciocalteu procedure (Hartree, 1972).

3.2.3. Gel filtration

Gel filtration was performed by the method of Andrews (1964, 1965).

3.2.4. Oxidation and reduction of ovorubin and helicorubin

Oxidation and reduction of proteins were initially carried out by addition of K ferricyanide and Na dithionite respectively. In later experiments these were replaced by 1 mM Na persulphate and 1 mM dithiothreitol which, in short-term experiments, had analogous effects and the advantage of low absorbance at the wavelengths under investigation. Prolonged exposure to these substances leads to denaturation of the protein.

3.2.5. Carbon monoxide binding

This was studied as described in 2.2. Addition of reducing agent before and after exposure to CO does not affect the binding of this gas to ovorubin, which is in contrast to the behaviour encountered with CO-binding cytochromes, where the gas interacts with the haemoprotein in the reduced state.

All spectra were recorded with a Pye Unicam SP800 or SP8100 spectrophotometer in a cell of 1 cm light path unless stated otherwise.

3.2.6. Electrophoretic separation

Electrophoretic separations were carried out on acrylamide gels using the Pharmacia GE4 electrophoretic apparatus.

A 30% (w/v) stock solution was prepared in which the monomeric:dimeric acrylamide ratio was 29:1.

The nomenclature of Hjerten (1962) is used to describe gels, i.e. %T = g of acrylamide plus g of bis-acrylamide per 100 ml of solution. The cross-linking agent tetramethylethylenediamine (TEMED) and the catalyst Na persulphate were obtained from Koch-Light and their final concentrations were kept at 0.15% (w/v) and 0.1% (w/v) respectively. Analytical gel electrophoresis was carried out either on gel rods or gel slab cassettes using well-formers. They were prepared according to the instructions supplied with the Pharmacia gel slab casting apparatus GSC 8. Format used: 8.2 x 8.2 x 0.27 cm.

For preparative purposes, slabs were prepared without sample wells. Up to 10 mg protein was applied per gel slab.

For anodic proteins, the following system was used:
Gel buffer: 0.75 M Tris-HCl, pH 8.8
Compartment buffer: 0.025 M Tris-HCl + 0.192 M glycine, pH 8.3.

The sample was dissolved in water containing 10% (w/v) sucrose and 5 $\mu g\ ml^{-1}$ bromophenol blue. 10-50 µl of sample containing up to 250 µg protein was applied by means of a Finn pipette to the top of a gel which was covered with compartment buffer. It was found that this produced sharper bands than the use of a sample in incubation buffer containing 0.125 M Tris-HCl, pH 6.8, which is commonly employed. It should also reduce the possibility of protein-buffer interaction.

For cathodic proteins, the method described by Gabriel (1971) was used. Although the account of this method described the tracer dyes methyl green and methylene green as synomymous, these are chemically different compounds, and it was found that only methylene green could be employed satisfactorily since:

a) Methyl green is decolourised, presumably by Na persulphate present in the gels, and cannot be used in discontinuous systems,
b) when used in a continuous buffer system, (after pre-electrophoresis) this dye shows two electrophoretically distinct components.

A solution of 10% (w/v) sucrose in water containing 50 $\mu g\ ml^{-1}$ methylene green was used instead of a sample incubation buffer for reasons given above.

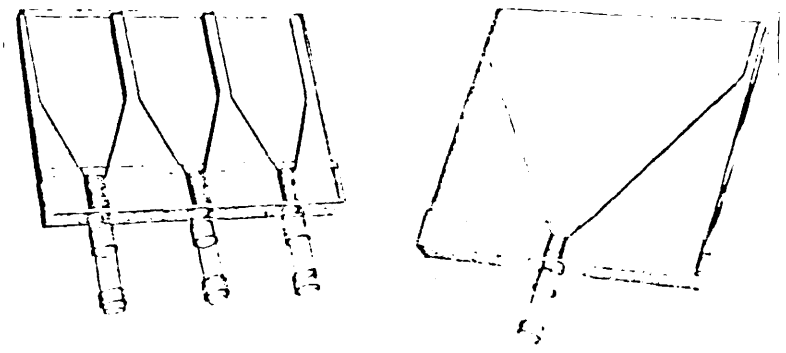
Gel and compartment buffers were prepared as described by Gabriel (1971).

Gel buffers were diluted 1:1 before use with the stock acrylamide solution to produce a 7.5% gel. All solutions were deaerated before polymerisation.

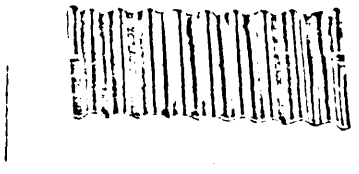
Electrophoresis was carried out at 8-10°C at constant current; 1 mA per gel rod or 4 mA per gel plate was applied for the first 30 min. of every run. The current was then doubled until the tracer dye had migrated 7.8 cm. The positions of the dye were marked with india ink and the gels were fixed and stained for protein for 60 min. in a solution containing 1.25 g Commassie brilliant blue and 50 g TCA dissolved in 50% (v/v) methanol and 7.2% (v/v) acetic acid. The solution was filtered before use.

For the detection of protein-bound carbohydrates, the periodic acid Schiff (PAS) staining procedure was carried out as described by Clarke (1964) following fixation for 1 hr in 10% (w/v) TCA. The excess Tris in gels was leached out with 7% (v/v) acetic acid followed by a 2 hr wash in distilled water.

Destaining was carried out electrolytically in a Uniscil electrophoretic de-stainer UDSI using 10% (v/v) methanol-acetic acid containing 2% glycerol for protein stains, and



Triangular section cells



Gel cutter

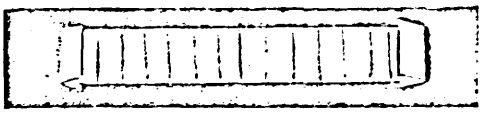


Fig. 3.2.1. Equipment for preparative electrophoresis

1% Na metabisulphite in 7% (v/v) acetic acid for protein-bound carbohydrate, until all background stain was removed. Current of the order of 1.5-2 A was supplied by a car battery charger.

3.2.7. Preparative electrophoresis

The first steps in preparative gel electrophoresis were carried out on gel plates as described above. Since the gels were polymerised with Na persulphate, this necessitated a preliminary run of *ca.* 45 min. to elute the oxidising agent from the gel. During this time buffer circulation was stopped and the buffer was subsequently replaced by fresh buffer.

Up to 10 mg protein was applied to each gel plate. At the end of every run, 0.5 cm of the right- and left-hand sides of the gel were removed with a gel-cutter, fixed, stained for protein, destained and aligned to the main gel plate which had been kept in a cold moist atmosphere. Staining and destaining were attended by slight volume changes with respect to the untreated gel. Complementary alignment could nevertheless be achieved with reasonable accuracy because the gel cutter produced an undulating pattern. The unstained protein bands could then be removed with a gel slicer.

In this particular case, identification of the bands was greatly facilitated by the native colour of the proteins. The unstained gel section containing the protein under investigation was then transferred to a cell of triangular section obtained from Uniscil (UPAI). The base of the cell was fitted with a tube terminating in a dialysis membrane and the neck blocked with rubber foam to prevent gel particles collecting on the membrane. For details of equipment, see Figure 3.2.1. This electrodialysis cell fits into the Pharmacia electrophoresis apparatus GE-4. The protein was released from the gel by electrophoresis in the appropriate buffer system and carried on to the dialysis membrane for collection. Current up to 1 mA per cell was applied at 10°C until the gel was colourless. The eluted protein was removed from the dialysis membrane, dialysed, freeze-dried and further examined by re-electrophoresis, electrophoresis on gradient

gels and by SDS-PAGE electrophoresis as well as by spectrophotometry.

3.2.8. Gradient-gel electrophoresis

Gradient-gel electrophoresis of the purified proteins was carried out on Pharmacia polyacrylamide gradient gels PAA 4/30 and PAA 2/16. For anionic proteins, the procedure recommended by Pharmacia was followed. For cationic proteins, the method of Gabriel (1971) was used with an equilibrium run for 45 min. at 125 V, and a total running time of 15 hrs at 125 V constant voltage. 5 µl samples containing 20-30 mg protein/ml were applied. Separation was achieved with the help of a plastic sample spacer, (see Fig. 3.2.1.) which divided the flat gel surface into 14 discrete compartments. (The variety supplied by Uniscil was preferable to that from Pharmacia, the latter having a tendency to come away from the gel, with consequent mixing of the samples).

Fixation and staining for protein and carbohydrate were carried out as earlier described.

3.2.9. Polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulphate (SDS) gels

The mobilities of most proteins in SDS-PAGE are linearly related to the logarithms of their molecular weights. The method is, however, known to produce erroneous molecular weight values for many polypeptides with carbohydrate prosthetic groups. Since ovorubin contains ca. 20% carbohydrate and in view of the uncertainty of molecular weight determinations for glycoproteins, it was decided to evaluate two molecular parameters that together largely determine the rate of migration in any electrophoretic system (Banker and Cotman, 1972).

M_o : free electrophoretic mobility

This describes migration in the absence of a sieving medium, and is a function of molecular size and charge.

K_R : retardation coefficient

This is solely dependent on molecular size and relates this to the sieving properties of the medium.

Both quantities can be measured during SDS gel electrophoresis

by determining migration rates in gels of different acrylamide concentrations based on the equation proposed by Ferguson (1964):

$\log M = \log M_o - K_R T$, where

M = electrophoretic mobility in a gel with percentage of acrylamide = T.

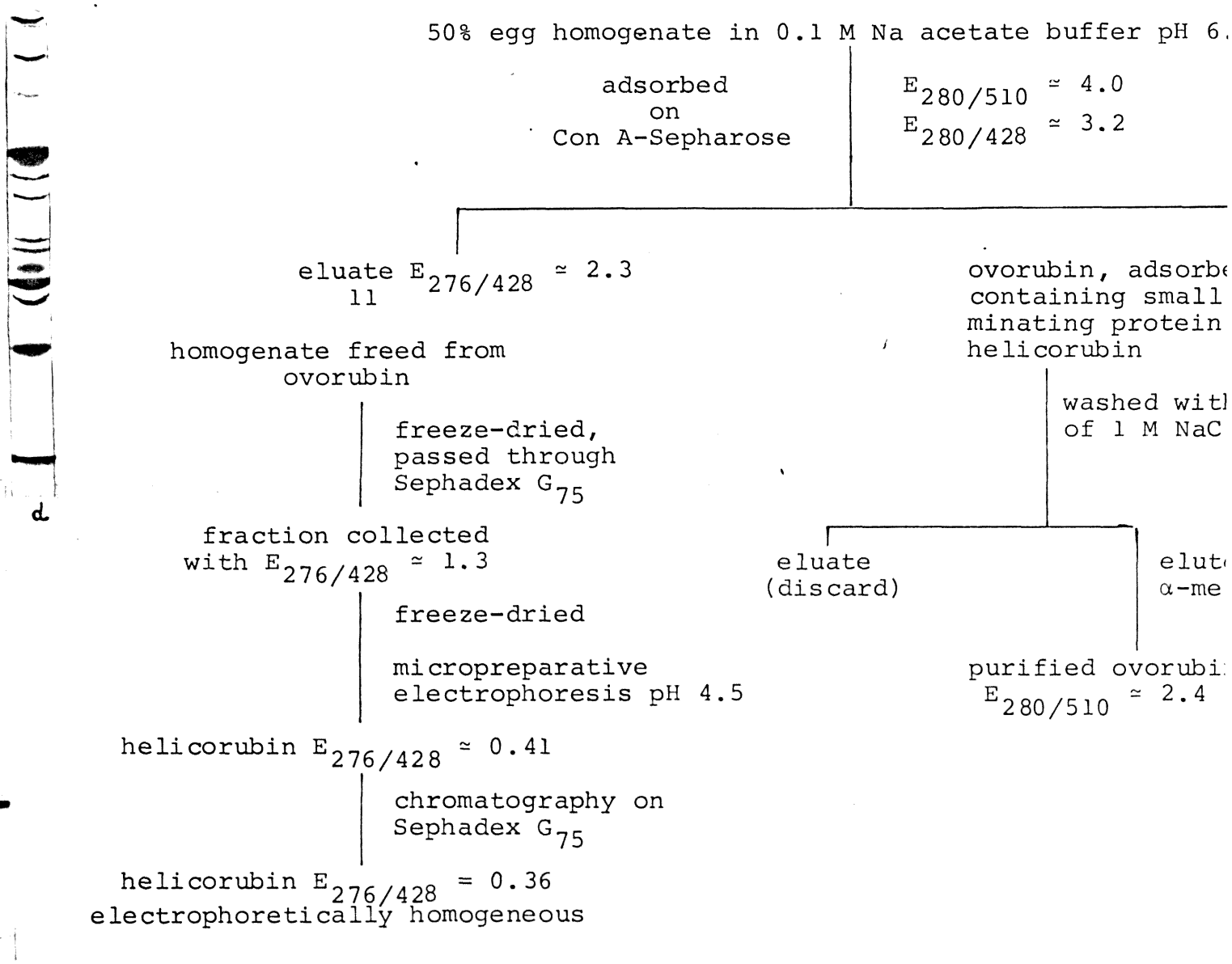
For convenience, the relative electrophoretic mobility, R_F, is measured; "Ferguson plots" of log R_F versus T are linear with a slope K_R and intercept log M_o at T = 0.

It has been shown (Banker and Cotman, 1972) that measurements of K_R and M_o for standards and the protein under investigation provide a useful check for possible anomalies, since plots of M_o versus K_R, which can be constructed without prior knowledge of molecular weight, reveal any unusual behaviour. Neville (1971) showed that measurement of retardation coefficients could be used to estimate the molecular weights of soluble proteins, and concluded that it should be possible to use it in the determination of molecular size of 'anomalous' proteins; the use of these coefficients essentially corrects for any unusual free mobility, which could explain anomalous rates of migration, as observed with some glycoproteins.

A discontinuous buffer system for molecular weight determination was chosen because of its improved resolution due to SDS-protein stacking in sharp starting zones. However, under the same conditions, SDS is also stacked, leading to inordinately wide starting zones and thus impaired resolution. This zone-broadening makes the trailing edge of the stack and the tracking dye migrating there, e.g. BPB at low gel concentration, unsuitable as a valid reference for R_F, while the leading edge of the stack and the tracking dye migrating there, e.g. pyronin Y-SDS, are a valid R_F reference. At high T both SDS and pyronin Y-SDS unstack, whereas BPB now moves to the leading edge of the stack and becomes, for that T range, a valid reference for R_F (Wyckoff *et al*, 1977).

Since at the intermediate transient state BPB increasingly advances from the trailing edge into the stack, while pyronin Y

Fig. 3.2.2. Purification scheme of ovorubin and helicorubin from homogenate



recedes increasingly from the leading edge, both tracer dyes were employed in each electrophoretic run.

Buffer system used:

Gel buffer: 0.75 M Tris-HCl, 0.2% SDS, 8 M urea, pH 8.8.
Sample incubation buffer: 0.125 M Tris-HCl, 4% SDS, 10% mercaptoethanol, 8 M urea, 10% sucrose, pH 6.8.
Compartment buffer: 0.025 M Tris-HCl, 0.192 M glycine, 0.1% SDS, pH 8.3.

Standards used:

Molecular weight markers for SDS-PAGE, BDH product No. 44223 2U. MW range 14.300-71.500. 1 ml of sample incubation buffer and 1 ml of water were added to the contents of one vial and shaken until solution was complete. Aliquots were then incubated at 100°C for 2 min. and loaded on to the gel.

1-3 mg of test protein was treated in the same way as the standard molecular weight markers.

Fixation and staining for protein and carbohydrate were carried out as described earlier.

A purification scheme for ovorubin and helicorubin from egg homogenates centrifuged at 2,000g is shown in Fig. 3.2.2.

Fig. 3.2.2.

a) Electrophoretic pattern of the egg homogenate in a β-alanine-acetic acid buffer, pH 4.5.

b) The same in a Tris-glycine buffer, pH 8.3.

c) The same, SDS-electrophoresis in a 10% gel, Tris-glycine buffer, pH 8.3.

d) Homogenate freed from ovorubin, SDS-Electrophoresis in a 10% gel. Tris-glycine buffer, pH 8.3.

e) Helicorubin, SDS electrophoresis in a 10% gel, Tris-glycine buffer, pH 8.3.

f) Electrophoretic pattern of ovorubin in a β-alanine-acetic acid buffer, pH 4.5.

g) The same in a Tris-glycine buffer, pH 8.3.

h) The same, SDS-electrophoresis in a 10% gel, Tris-glycine buffer, pH 8.3.

Prep No.	Values for 5% egg homogenates in 0.1 M Tris-HCl buffer, pH 7.0 E_{280}	E_{428}	E_{510}	purified OR $E_{280/510}$	mg of protein per gram of eggs ovorubin	helicorubin	total protein	OR + HR % of total protein	molar ratio OR : HR	Homog. $E_{280/510}$	% recovery OR	HR
1	2.34	0.765	0.642	2.46$^{\Phi}$	28.4	1.38	50.04	59.5	1:1.23	3.64	69.5	
2	2.67	0.89	0.621	2.67$^{\Phi}$	29.5	1.64	60.44	51.5	1:1.42	4.29	71.3	
3	2.95	0.83	0.567	2.86$^{\Phi}$	29.0	1.51	64.87	47.0	1:1.32	5.2	72.4	
4	2.4	0.745	0.634	2.39^{+}	27.2	1.34	52.69	54.2	1:1.25	3.8	71.9	
5	2.38	0.793	0.643	2.27^{+}	26.36	1.4	53.05	52.33	1:1.35	3.7	72.3	
6	2.31	0.767	0.665	2.31^{+}	27.79	1.38	48.75	59.8	1:1.26	3.47	68.4	
7	2.71	0.871	0.669	2.62$^{\Phi}$	31.6	1.6	58.24	57.0	1:1.29	4.05	74.8	30
8	2.79	0.825	0.648	2.8$^{\Phi}$	32.8	1.51	59.2	57.96	1:1.17	4.3	73.2	32
9	2.31	0.798	0.62	2.27^{+}	25.36	1.45	49.63	54.0	1:1.45	3.72	72.7	35.2
10	2.27	0.731	0.663	2.05^{+}	24.57	1.31	48.73	53.11	1:1.35	3.42	70.5	32.8
11	2.52	0.731	0.566	2.75$^{\Phi}$	28.0	1.31	54.1	55.2	1:1.2	4.45	71.3	36.1
12	2.48	0.771	0.59	2.27^{+}	24.17	1.39	54.04	47.67	1:1.46	4.2	68.7	35.6
mean	2.51	0.793	0.627	2.476	27.89	1.45	54.5	53.2	1.31	4.019	71.4	33.6
St. Dev.	0.22	0.052	0.0359	0.257	2.62	0.116	5.15	4.23	0.096	0.505	1.89	24

Table 3.3.1.

+ ovorubin prepared by adsorption on Con A-Sepharose

Φ ovorubin prepared by adsorption on calcium phosphate gel

3.3. Results

3.3.1. Purification of ovorubin and helicorubin P from egg homogenates of *P. canaliculata*

The results of twelve purifications of ovorubin and six of helicorubin by the method shown in Fig. 3.2.2. are given in Table 3.3.1.

Concentrations of ovorubin and helicorubin in 5% (w/v) egg homogenates were estimated from measurements at 510 nm and 428 nm (dithionite-reduced homogenate). Calculations were made on the basis of an absorbance for ovorubin of $E_{280}^{0.1\%} = 1.00$, and the $E_{280/510}$ ratio of ovorubin purified from the homogenate in question.

For helicorubin P, $E_{428}^{0.1\%}$ was taken as 9.65.

Corrections for the E_{510} absorbance were based on $E_{510}^{0.1\%} = 0.52$ for reduced helicorubin and an average estimated content of 1.4 mg helicorubin per gram of eggs. Similar corrections were made to account for co-absorbance of ovorubin at 428 nm. They were based on $E_{428}^{0.1\%}$ of 0.07 and an average ovorubin content of the order of 28 mg gr^{-1} eggs. Such calculations involve an error of approximately 5-7%.

The values for the ovorubin content based on the $E_{280/510}$ ratio of the protein purified by the method of Cheesman and on the absorbance at 510 nm for the egg homogenate are probably too high, since such preparations have been shown to be contaminated by a polysaccharide which gives rise to turbidity in the UV range.

3.3.1.a Purification of ovorubin

Ovorubin eluted from concanavalin A-Sepharose with 0.3 M α-methylglucoside or α-methylmannoside accounted for ca. 65% of the total ovorubin. There was, however, still ovorubin bound to the lectin which could not be recovered even by prolonged incubation with increasing concentrations of either eluting agent. A further 5-7% could be removed by treatment with 0.05 M Na borate buffer, pH 6.0. The fraction eluted with borate was usually associated with an increased $E_{280/510}$ ratio. However, some 25% of the ovorubin adsorbed onto the

lectin could not be eluted even on overnight washing with borate buffer. Ovorubin was subsequently dialysed for five days at 2-5°C against 50 ℓ distilled water. By this time about 3% of the total ovorubin had come out of solution and could be collected by centrifugation. This fraction of ovorubin is insoluble in water, but dissolves quite readily in e.g. 0.1 M Tris-HCl buffer pH 7.0.

As can be seen from Table 3.3.1., ovorubin prepared by the method of Cheesman constantly gives rise to a preparation with a higher $E_{280/510}$ ratio. It is also much more sensitive to extensive dialysis and, under otherwise identical conditions, up to 40% of the total ovorubin comes out of solution, but can be redissolved in buffer.

In every case, the supernatant after extensive dialysis showed an improved $E_{280/510}$ ratio, while the redissolved precipitate had an increased ratio.

When the water-insoluble precipitate was dissolved in M NaCl and the protein adsorbed on Con A-sepharose, a white turbid eluate of strong UV absorbance was obtained. This is insoluble in water after extensive dialysis and chromatography on Sephadex G200 indicates an apparent molecular weight in excess of 2×10^6. It gives a negative biuret reaction and a positive Molisch test.

Electrophoretic analysis of ovorubin prepared by these different methods in 7.5% polyacrylamide electrophoresis shows that, despite spectral differences, all give rise to similar electrophoretic patterns. At pH 8.3 and 4% gel strength four bands move towards the anode and one remains at the origin. At pH 4.5 two bands migrate towards the cathode and one remains at the origin. One component, that of highest mobility, predominates in each case. The patterns of sub-units obtained in SDS-PAGE are also identical.

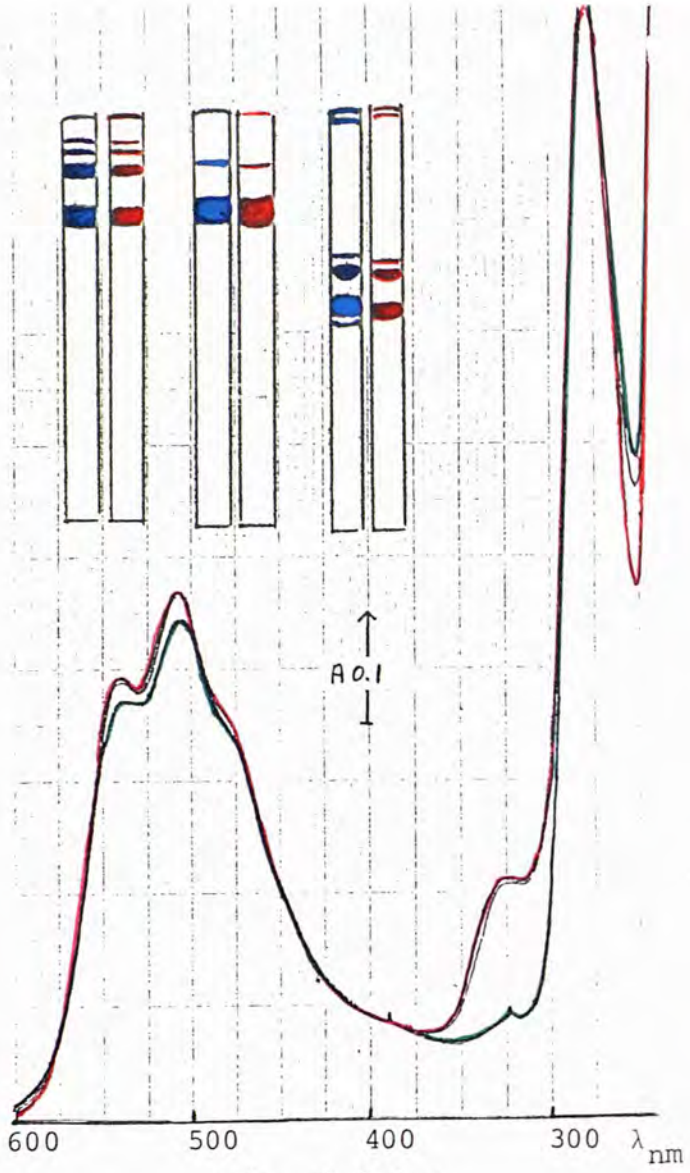


Fig. 3.3.1.a

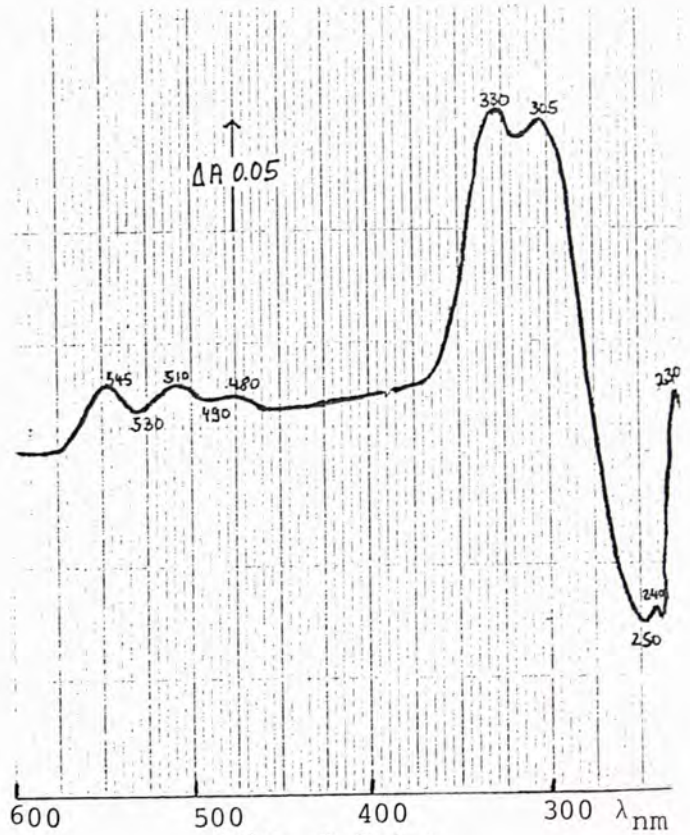


Fig. 3.3.1.b

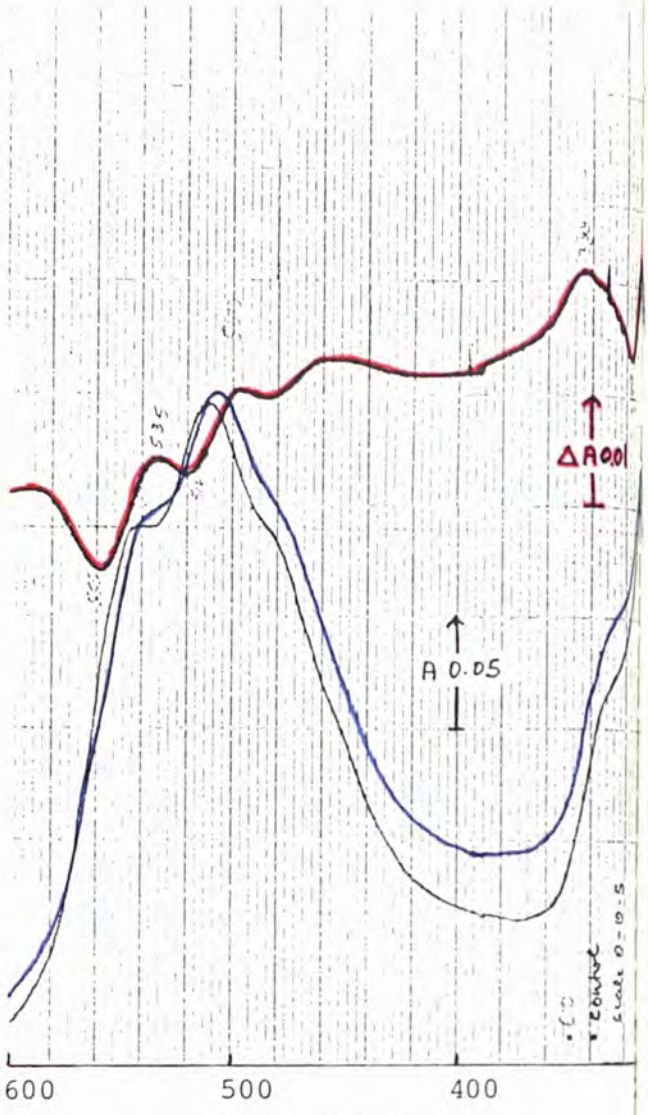


Fig. 3.3.1.c

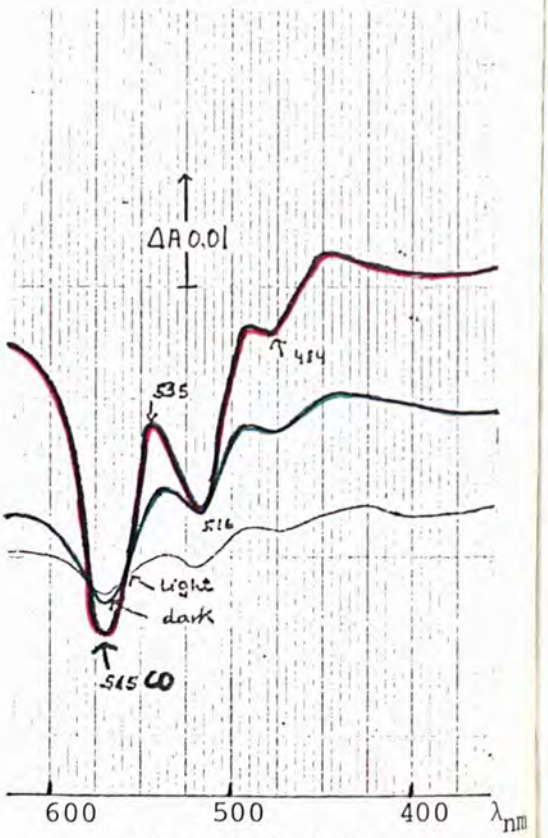


Fig. 3.3.1.d

Fig. 3.3.1.a Spectra of ovorubin purified on Con A-Sepharose, eluted with α-methylglucose, after dialysis and before freeze-drying (path length 1 cm)

- 1 mg ml^{-1} ovorubin in Tris-HCl buffer, pH 7.0
- the same, reduced with 1 mM Na dithionite, ($E_{280/510}$ = 2.21)
- the same, oxidised with 1 mM Na persulphate

Electrophoretograms from left to right show -

separation in Tris-HCl buffer, pH 8.3,
separation in β-alanine-acetic acid buffer, pH 4.5,
separation by SDS-PAGE in a 10% gel

- stained for protein
- stained for protein-bound carbohydrate

Fig. 3.3.1.b Difference spectrum of dithionite-reduced ovorubin against the persulphate-oxidised protein.

Fig. 3.3.1.c Effect of carbon monoxide on the spectrum of ovorubin (ca. 0.65 mg ml^{-1})

- control
- after 2 min. exposure to CO in the dark
- difference spectrum of ovorubin exposed to CO for 2 min. against untreated control

Fig. 3.3.1.d Effect of light on binding of carbon monoxide by ovorubin (ca. 0.65 mg ml^{-1})

- difference spectrum of ovorubin exposed to CO for 3 min. against untreated control
- the same, after 15 min. in the dark
- the same, after 15 min. exposure to light (5 W m^{-2})

For details see Materials and Methods 3.2.5.

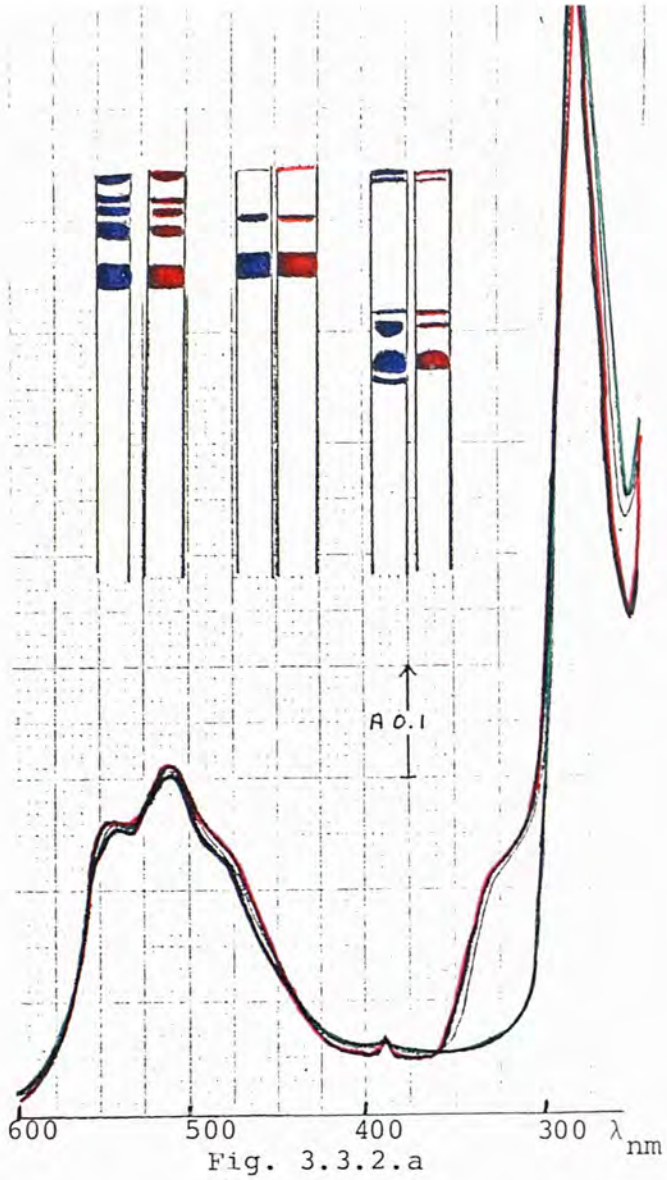


Fig. 3.3.2.a

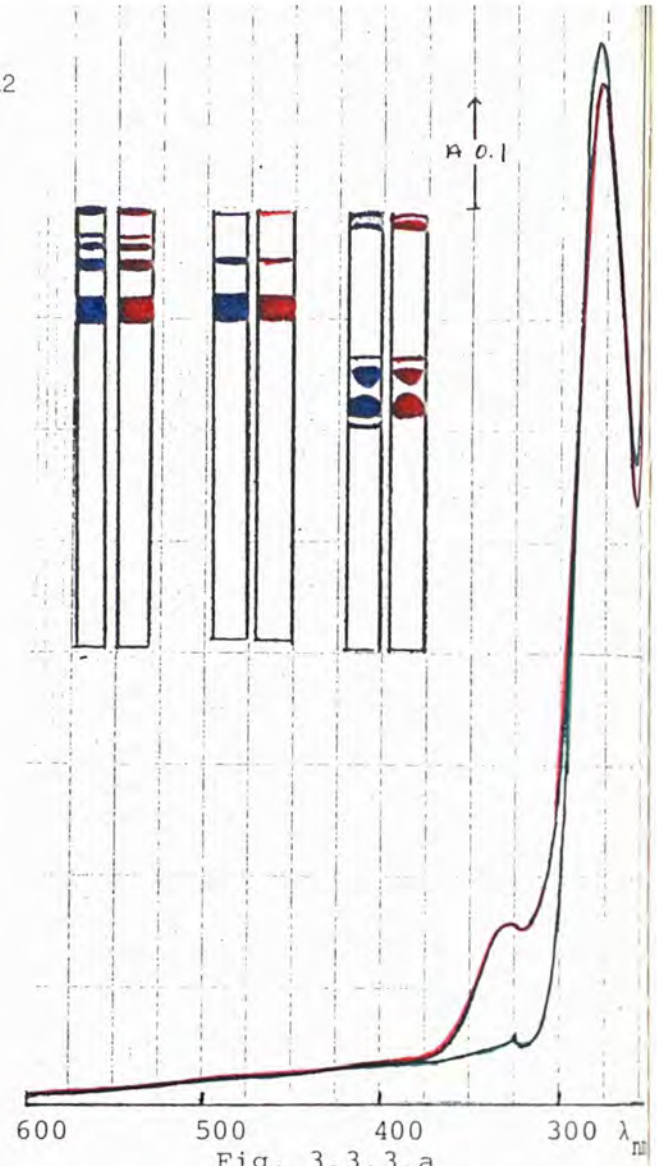


Fig. 3.3.3.a

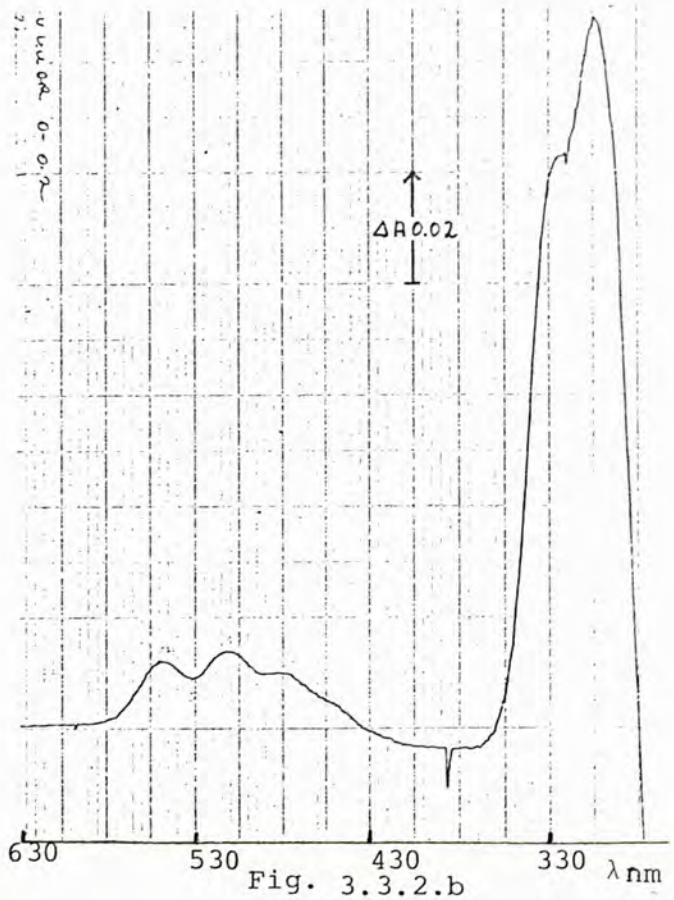


Fig. 3.3.2.b

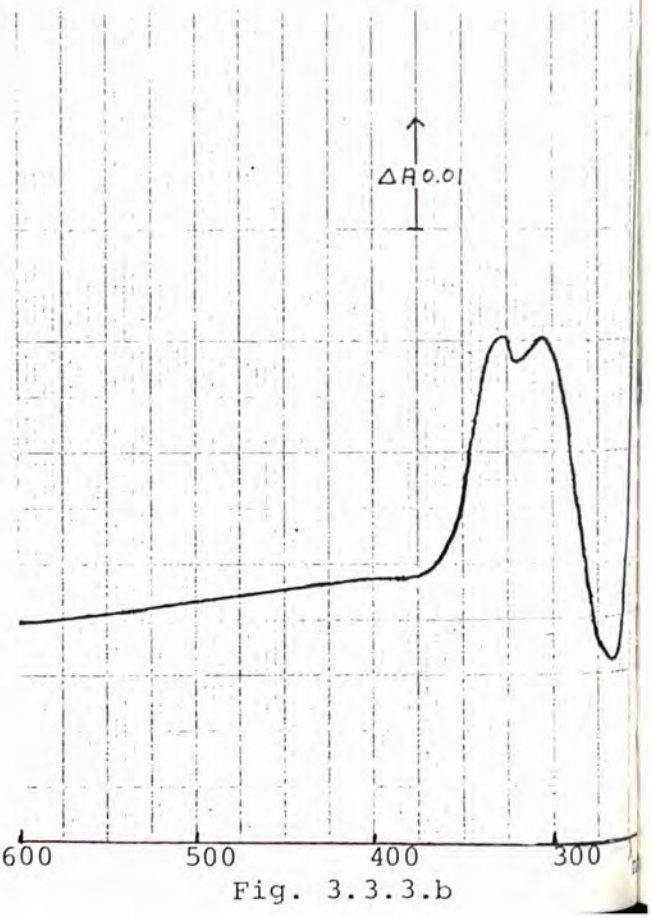


Fig. 3.3.3.b

Fig. 3.3.2.a Spectra of the ovorubin fraction removed with 0.05 M borate buffer from a column after elution with α-methylglucose. Measurements were made in a 1 cm cell after dialysis and before freeze-drying.

- 1 mg ml^{-1} ovorubin in Tris-HCl buffer, pH 7.0
- the same, reduced with 1 mM Na dithionite ($E_{280/510}$ = 3.12)
- the same, oxidised with 1 mM Na persulphate.

Electrophoretograms as for Fig. 3.3.1.a.

Fig. 3.3.2.b Difference spectrum of the dithionite-reduced against the persulphate-oxidised ovorubin

The CO-binding property of this fraction is indistinguishable from that already shown (Fig. 3.3.1.c).

Fig. 3.3.3.a Spectra of the apoprotein of ovorubin 0.8 mg ml^{-1} in the oxidised and reduced forms, clearly indicating that the inflexion in the 330 nm region of the spectrum is not associated with the carotenoid moiety

Electrophoretograms and experimental details as for Fig. 3.3.1.a.

Fig. 3.3.3.b Difference spectrum of the dithionite-reduced against the persulphate-oxidised apoprotein. The apoprotein shows no spectral changes in the visible region on exposure to CO.

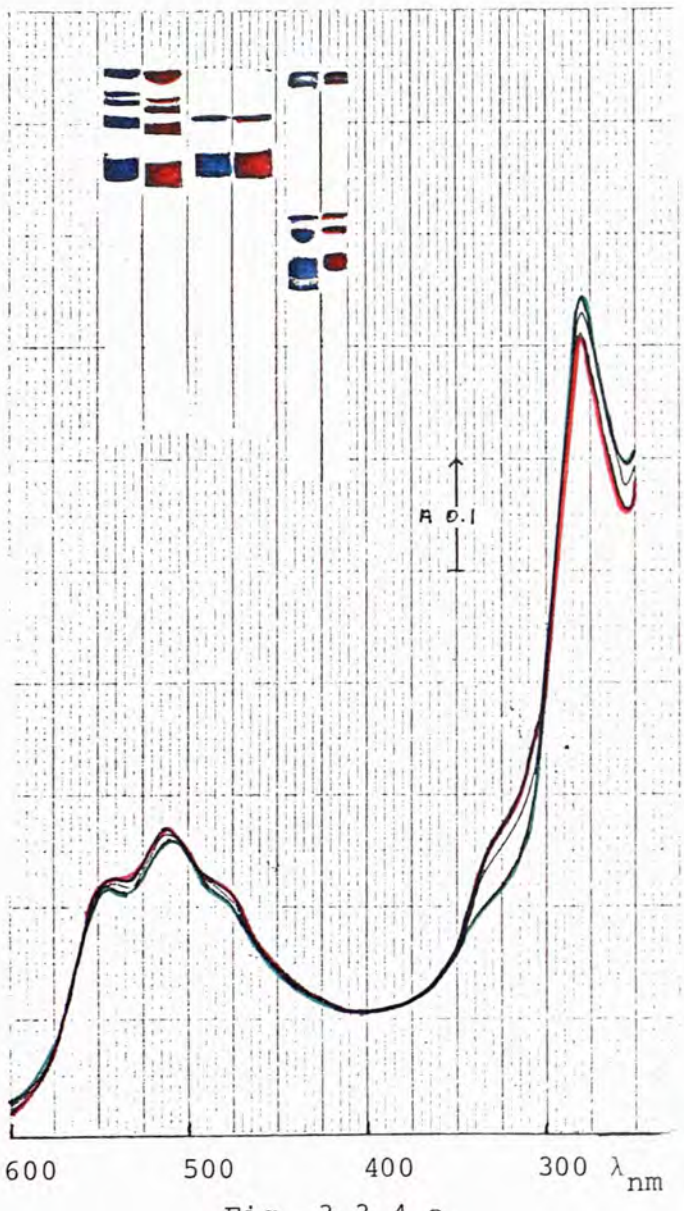


Fig. 3.3.4.a

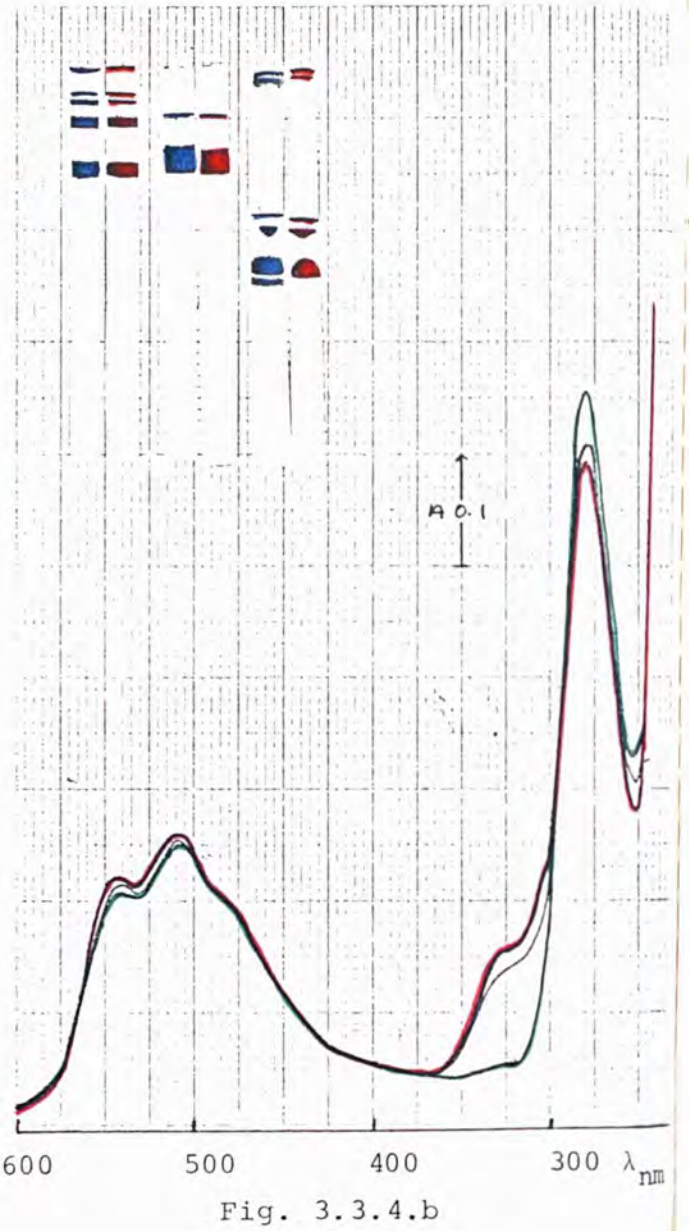


Fig. 3.3.4.b

Fig. 3.3.4.a Spectra of ovorubin purified by the method of Cheesman

- 0.6 mg ml^{-1} ovorubin in Tris-HCl buffer pH 7.0
- the same, reduced with 1 mM Na dithionite, $E_{280/510} = 2.77$
- the same, oxidised with 1 mM Na persulphate

Electrophoretograms as in Fig. 3.3.1.a.

Fig. 3.3.4.b Spectra of the same preparation of ovorubin, further purified by adsorption on Con A-Sepharose and elution with α-methylglucose

- 0.6 mg ml^{-1} ovorubin in Tris-HCl buffer pH 7.0
- the same, reduced with 1 mM Na dithionite, $E_{280/410} = 2.23$
- the same, oxidised with 1 mM Na persulphate

Electrophoretograms as in Fig. 3.3.1.a.

3.3.1.b Electrophoresis on gradient gels

Since there was a chance that the differences in migration rate for the ovorubin fractions were due solely to a difference in charge, as was found with hen's egg ovalbumin (Perlmann, 1950) this phenomenon was investigated by electrophoresis on gradient gels.

The results show that the four different components migrating into 4/30 gradient gels at pH 8.3 and the two at pH 4.5 are in fact of different molecular weight. Fig. 3.3.5. shows the relationship between molecular weight and the rate of migration on Pharmacia 4/30 and 2/16 gradient gels.

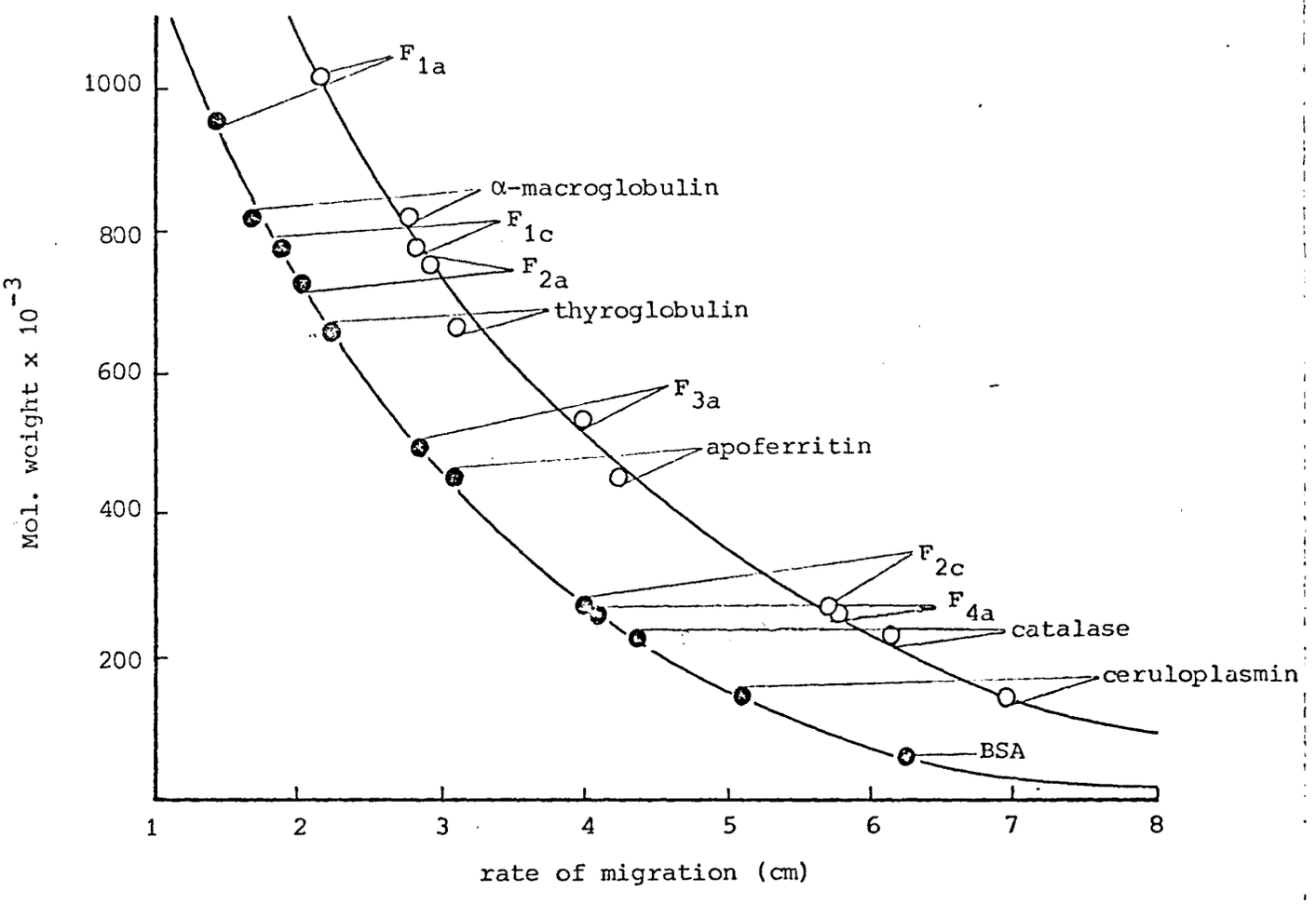

Mol. weight x 10^{-3}
1000
800
600
400
200
1
2
3
4
5
6
7
8
rate of migration (cm)
F_{1a}
α-macroglobulin
F_{1c}
F_{2a}
thyroglobulin
F_{3a}
apoferritin
F_{2c}
F_{4a}
catalase
ceruloplasmin
BSA

Fig. 3.3.5.

Fig. 3.3.5. The rate of migration of ovorubin on gradient gels

● 4/30

o 2/16

Table 3.3.2. summarises the data from which Fig. 3.3.5. was constructed. The results represent the mean values for three experiments.

pH	Fraction	4/30% gels		2/16% gels	
		Migration in mm	MW x 10^{-3}	Migration in mm	MW x 10^{-3}
8.3	F_o	origin	>1000	0.4	>1000
	F_{1a}	1.45	960	2.15	1010
	F_{2a}	2.05	730	2.95	759
	F_{3a}	2.9	500	4.05	530
	F_{4a}	4.15	280	5.85	270
4.5	F_{1c}	1.95	770	2.9	780
	F_{2c}	4.05	290	5.75	280

Table 3.3.2. Molecular weights of the fractions of ovorubin on gradient gels

The values obtained for molecular weight by this method do not represent exact multiples of the apparent monomeric form. Although all fractions contained bound carotenoid, it was possible that somewhat different molecular entities were concerned which had the common feature that they possessed an identical subunit involved in binding the carotenoid. This possibility needed further investigation; it was decided to purify the fractions by micropreparative gel electrophoresis at pH 4.5 and 8.3. These purified fractions were then further analysed by spectrophotometry, re-electrophoresis at pH 8.3 and 4.5 and SDS-PAGE.

3.3.1.c Molecular weight of ovorubin determined by chromatography on Sephadex G-200

Chromatography of calcium phosphate-purified ovorubin shows that it is eluted in the molecular weight range between 315,000 and 1,510,000. The elution pattern shows five peaks. The earlier mentioned macromolecule that gives rise to a positive

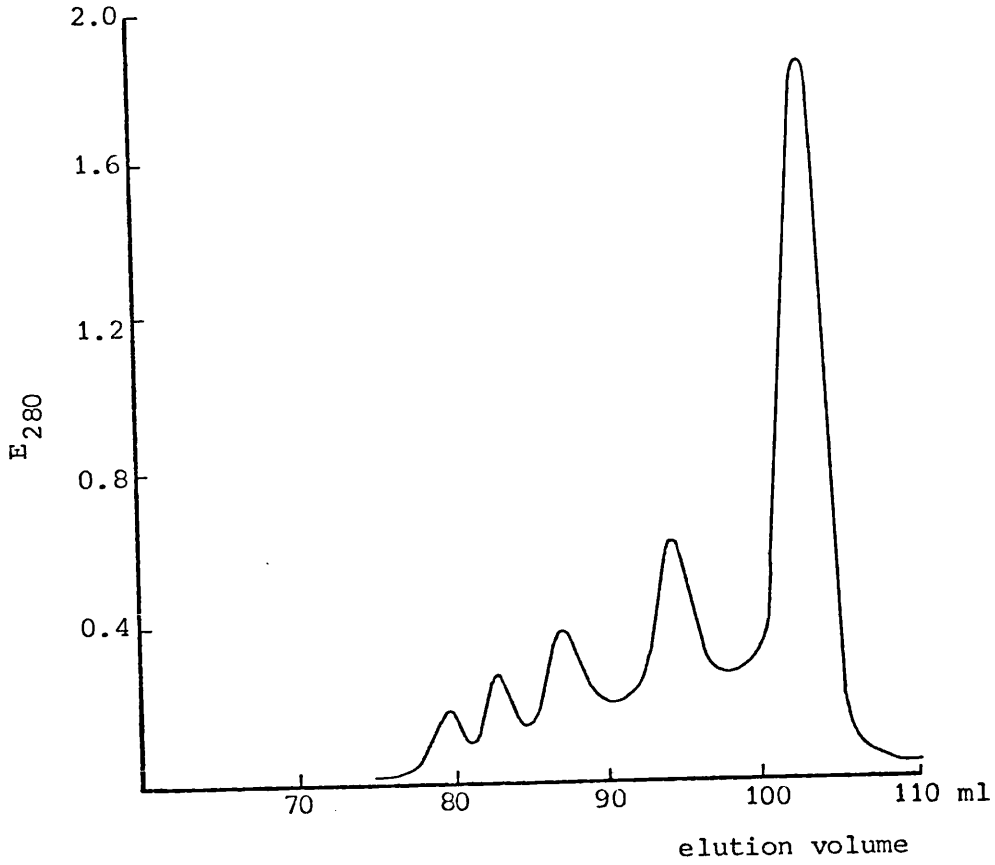


Fig. 3.3.6.

Molisch reaction can be separated from ovorubin under these conditions. Its elution volume closely resembles the void volume. The apparent molecular weight values, their $E_{280/510}$ and the appropriate percentage composition, as calculated from the areas under the peaks in Figs. 3.6, are shown in Table 3.3.3.

Fig. 3.3.6. Elution pattern of ovorubin prepared on calcium phosphate gel. Approx. 12 mg (dry weight) of ovorubin was applied to the column (length 41.5 cm, diameter 2.5 cm, elution volume 16.5 ml hr^{-1}) in 2 ml 0.1 M Tris-HCl buffer, M NaCl, pH 7.0.

peak	MW x 10^{-3}	$E_{280/510}$	% of total
1	1,510	3.4	3.5
2	1,100	2.87	5
3	850	2.61	10
4	580	2.28	19
5	315	1.92	62.5

Table 3.3.3.

Apparent mol weight, $E_{280/510}$ ratios, and the percentage ratios of the fractions obtained on Sephadex G-200.

3.3.1.d Ovorubin fractions obtained by micropreparative electrophoresis at pH 4.5 and 8.3

F_{2c}

This fraction, F_{2c}, shows a number of features distinctly different from those observed with cathodic fractions at pH 8.3. When frozen as an aqueous solution it turns yellow (Fig.3.3.7.b) and shows a broad peak between 420 and 430 nm.

On warming the fraction at 40°C for several hours, the characteristic ovorubin peak slowly reforms, although the sample is still very turbid and does not permit differentiation of spectral features in the UV range even after centrifugation at 12,000g for 30 min. (Fig. 3.3.7.b).

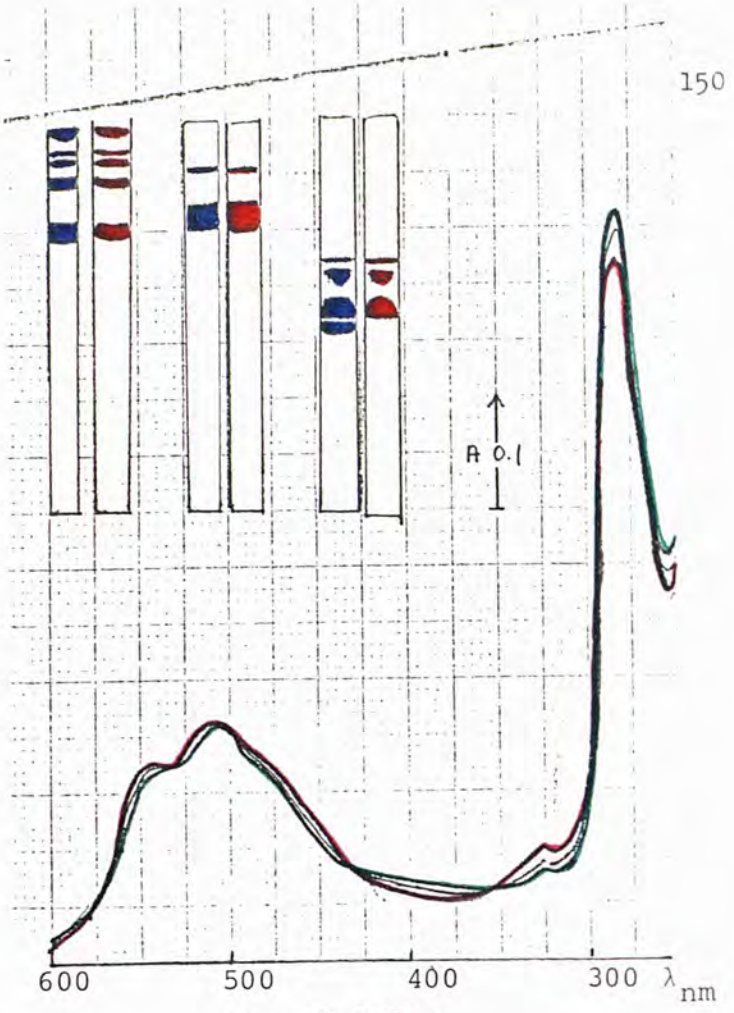


Fig. 3.3.7.a

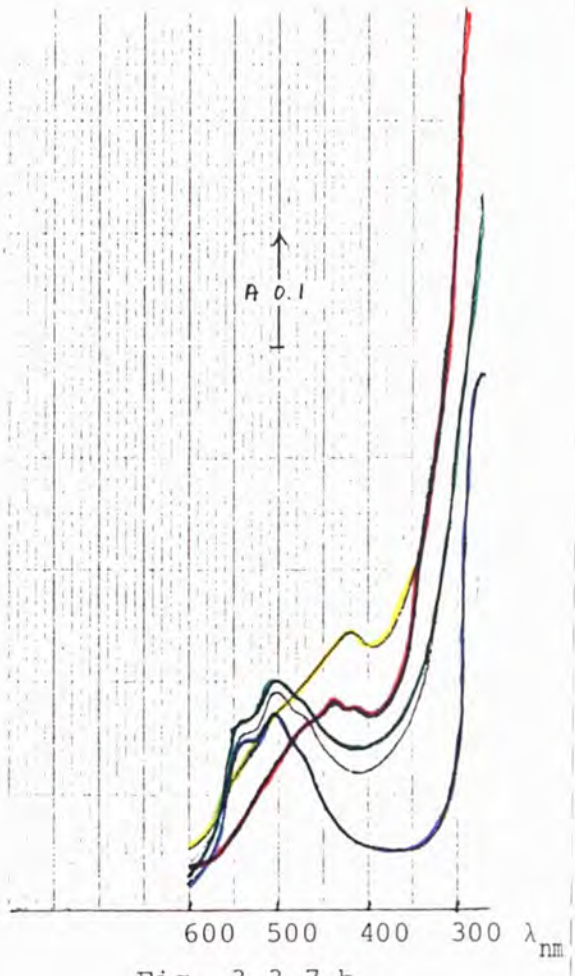


Fig. 3.3.7.b

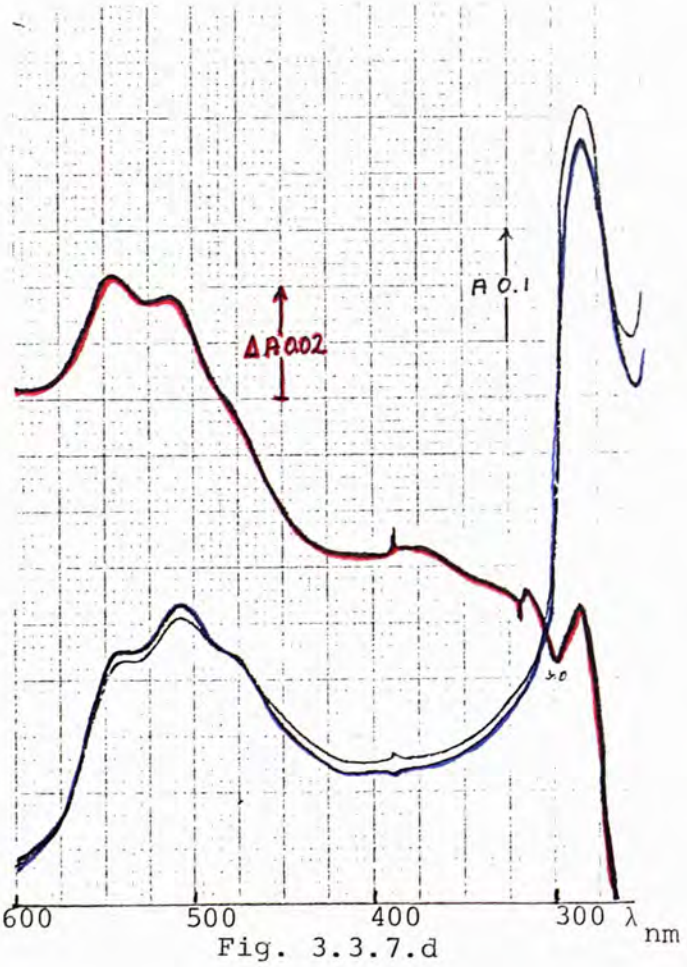


Fig. 3.3.7.d

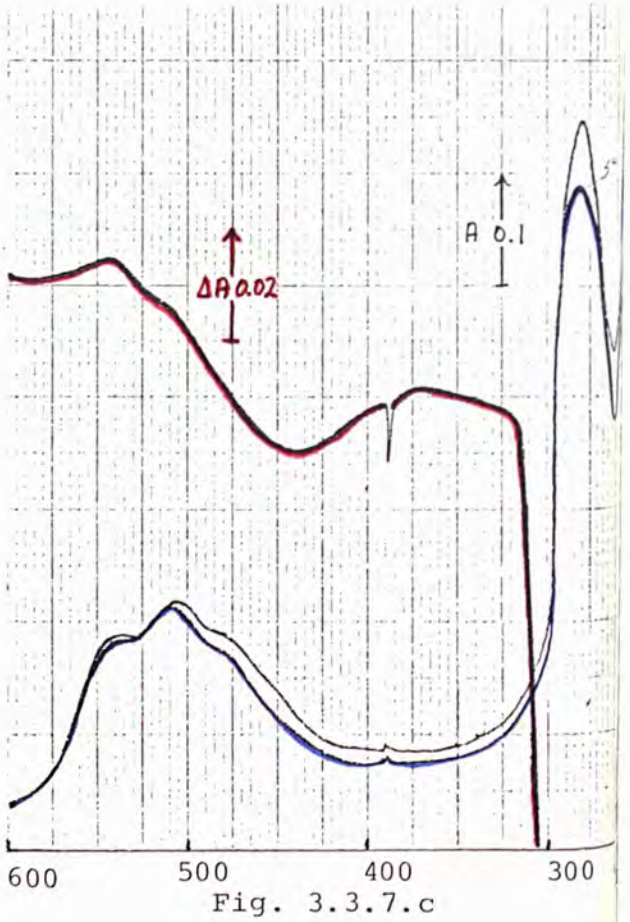


Fig. 3.3.7.c

Carbon monoxide-binding properties of this protein are different from those observed with anodic fractions immediately after dissolution in buffer (Fig. 3.3.7.c). After a period of 20 hrs in buffer, however, (Fig. 3.3.7.d) they approach those of the freshly prepared ovorubin and its anodic fractions.

Fig. 3.3.7.a Spectra of 0.65 mg ml^{-1} ovorubin F_{2c} redissolved after freeze-drying

- in 0.1 M Tris-HCl pH 7.0
- the same, reduced with 1 mM Na dithionite ($E_{280/510}$ = 2.95)
- the same, oxidised with 1 mM Na persulphate

Electrophoretograms as in Fig. 3.3.1.a.

Fig. 3.3.7.b Spectral changes observed on freezing F_{2c} in water

- cold-denatured F_{2c} (several days at -20°C) at pH 4.5
- the same after 1 hr at 40°C in 0.1 M Tris-HCl buffer, pH 7.0
- the same after 3 hrs at 40°C in 0.1 M Tris-HCl buffer, pH 7.0
- the same after 4 hrs at 40°C in 0.1 M Tris-HCl buffer, pH 7.0
- approx 0.45 mg ml^{-1} of F_{2c} (before freezing) in 0.1 M Tris-HCl buffer, pH 7.0

Fig. 3.3.7.c Effect of CO on F_{2c} freshly dissolved in 0.1 M Tris-HCl buffer, pH 7.0

- spectrum of 0.05 mg ml^{-1} F_{2c} (control)
- the same after 2 min. exposure to CO
- difference spectrum between CO-treated F_{2c} and control

Fig. 3.3.7.d The same as Fig. 3.3.7.c 20 hrs after dissolving F_{2c} in buffer

The fraction of lower mobility at pH 4.5, F_{1c}, shows cold-denaturation and CO-binding characteristics similar to those observed with F_{2c}.

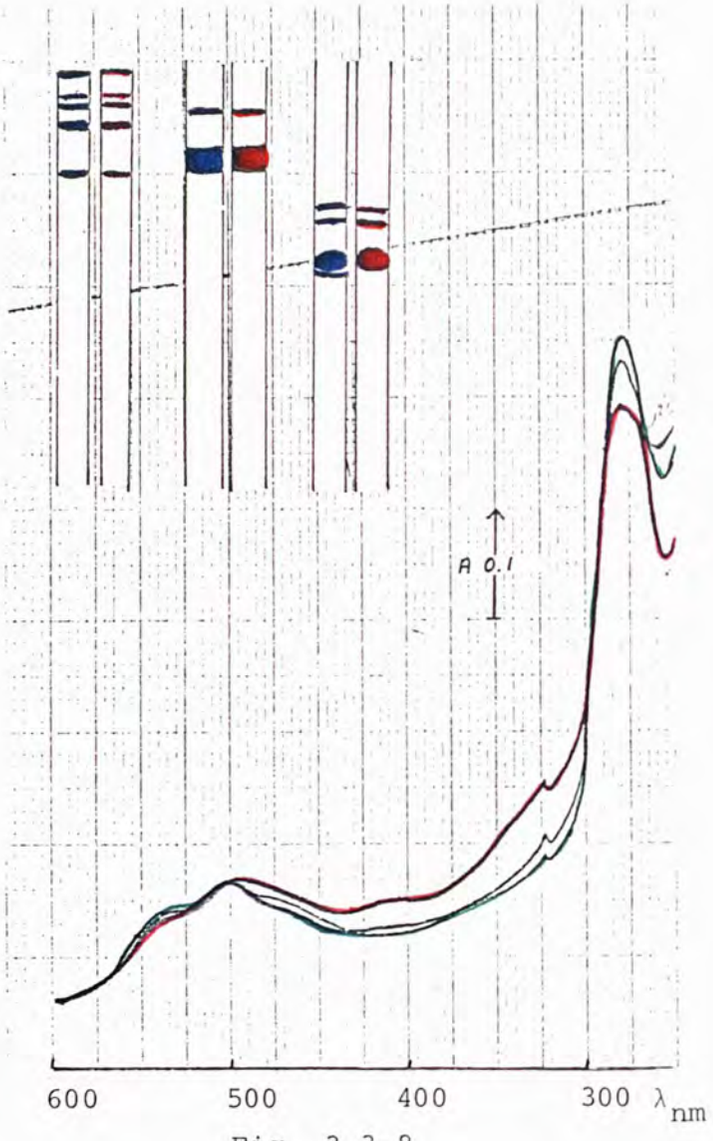


Fig. 3.3.8.

Fig. 3.3.8. The spectra of 0.65 mg ml^{-1} F_{1c}

- dissolved in 0.1 M Tris-HCl buffer, pH 7.0
- the same, reduced with 1 mM Na dithionite
- the same, oxidised with 1 mM Na persulphate

Electrophoretograms as above.

All other fractions of ovorubin migrate towards the anode at pH 8.3 or stay at the origin.

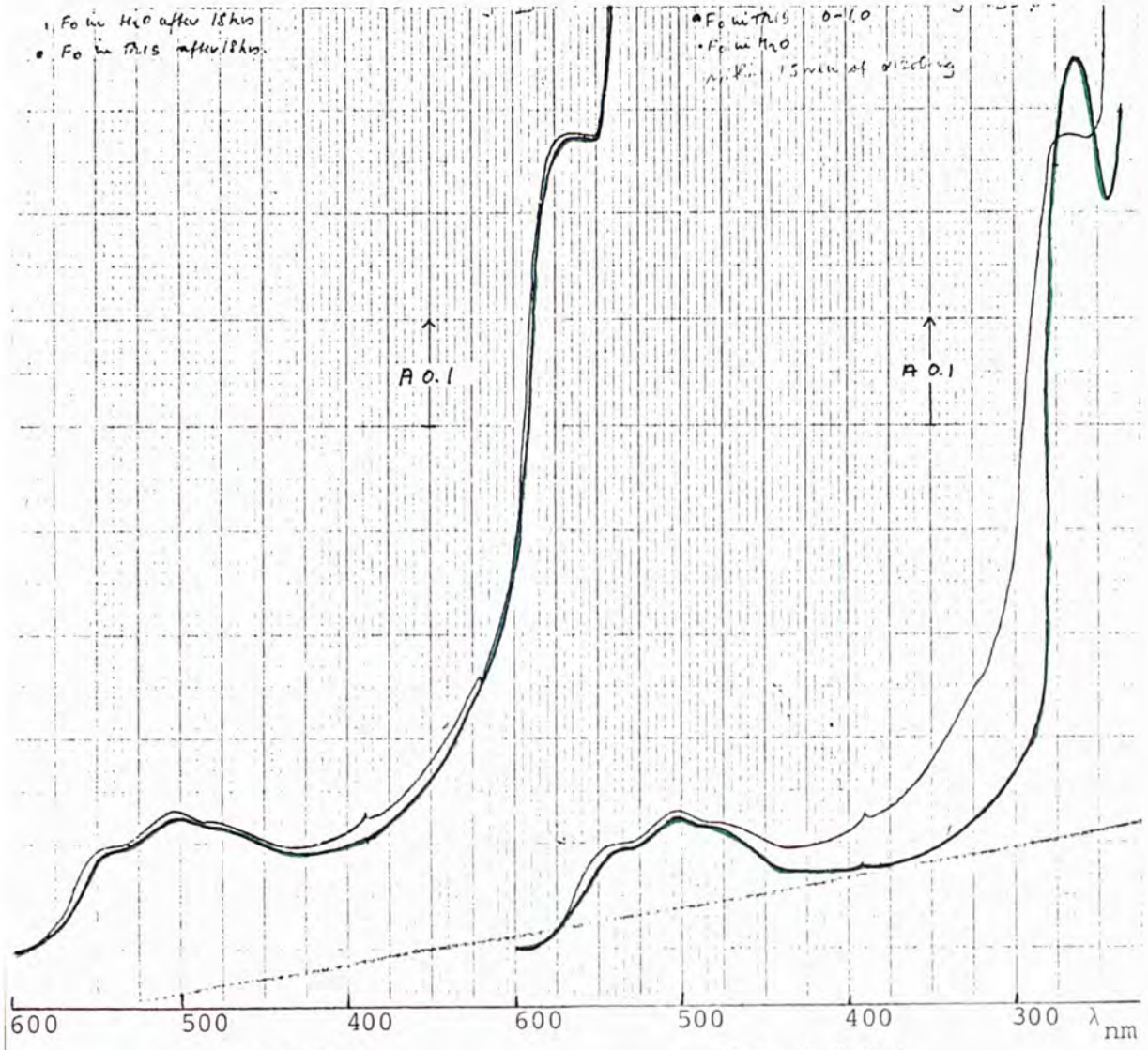


Fig. 3.3.9.c

Fig. 3.3.9.b

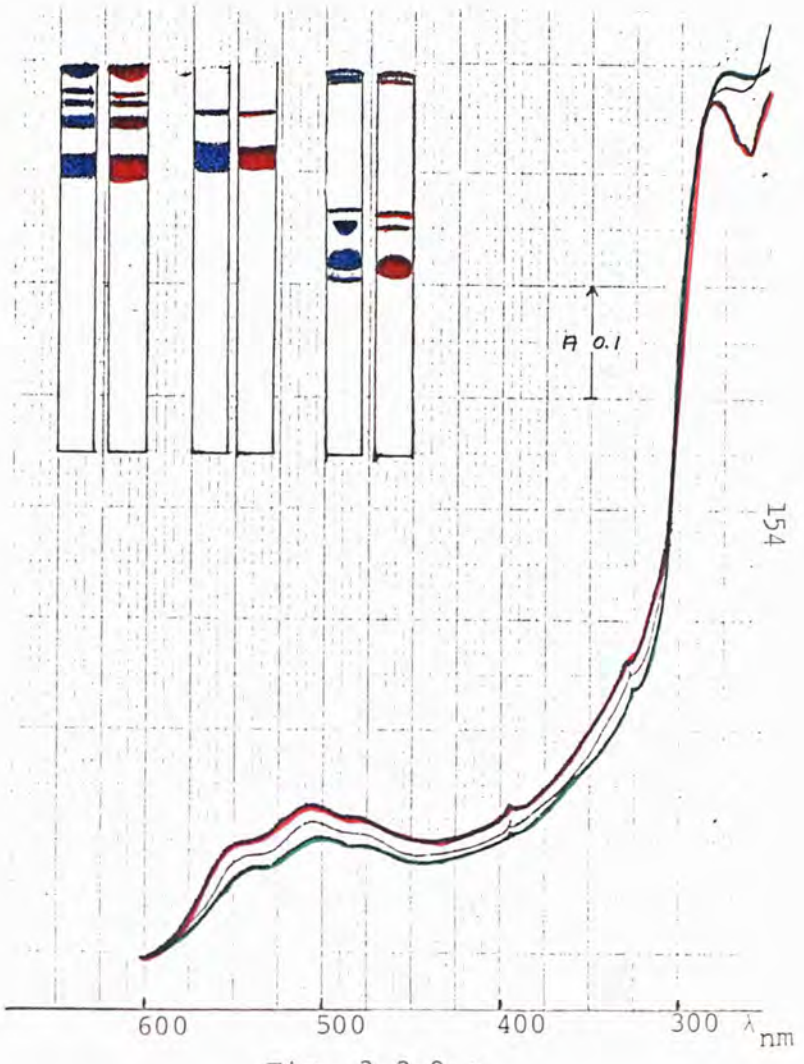


Fig. 3.3.9.a

Fig. 3.3.9.a The spectral and electrophoretic properties of the fraction remaining at the origin, F_{oa}, at 4% gel strength in Tris-HCl buffer, pH 8.3

- spectrum of approx. 0.4 mg ml^{-1} F_{oa} in Tris-HCl buffer, pH 7.0
- the same reduced with 1 mM Na dithionite
- the same oxidised with 1 mM Na persulphate

Fig. 3.3.9.b and c The spectral differences and changes occurring when this protein was dissolved in water and Tris-HCl buffer, pH 7.0, after 15 min. (9.b) and after 18 hrs (9.c)

- in water
- in Tris-HCl buffer pH 7.0

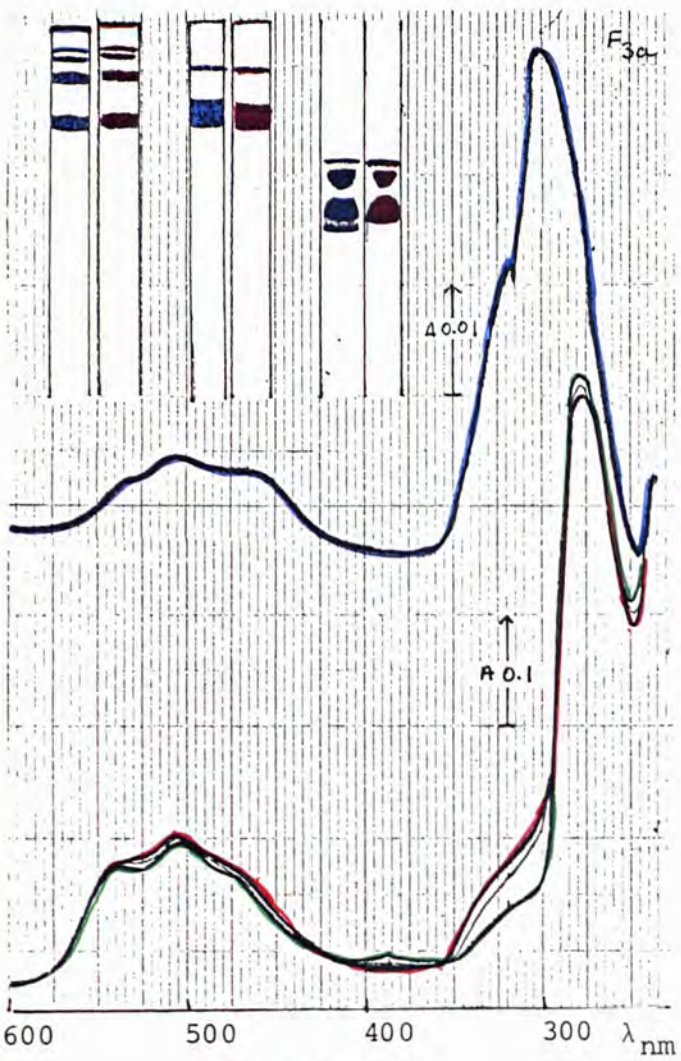


Fig. 3.3.12.

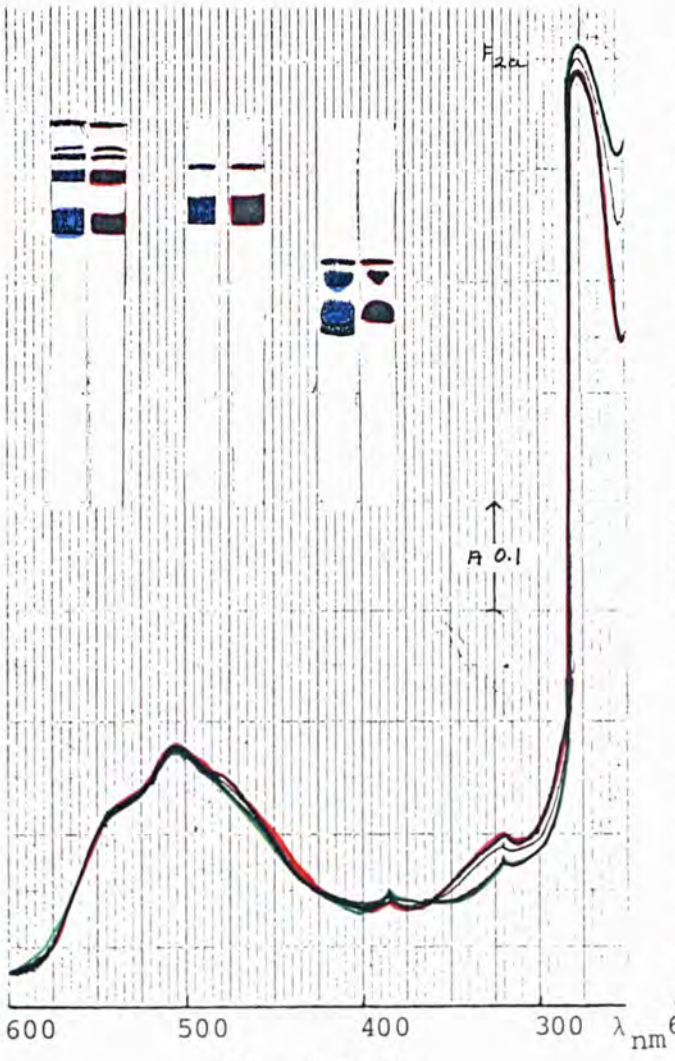


Fig. 3.3.11.

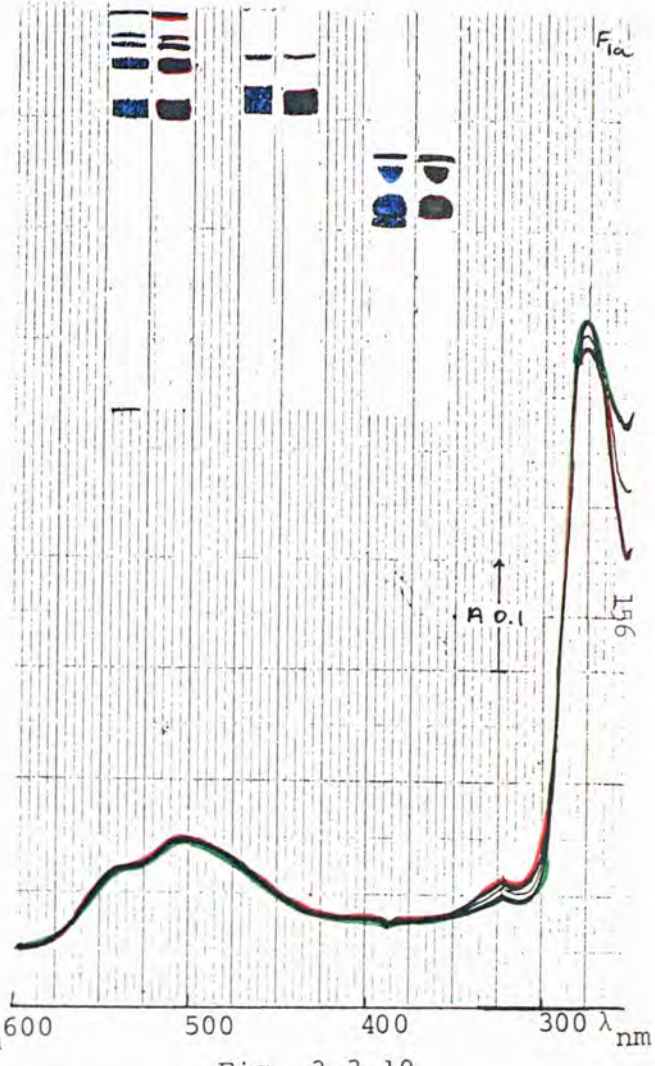


Fig. 3.3.10.

Figs. 3.3.10. and 3.3.11. The spectra and electrophoretic mobilities of the fraction of lowest mobility, F_{1a}, and that of intermediate mobility, F_{2a}, at pH 8.3 in

. Tris-HCl buffer system, pH 7.0
. the same, reduced with 1 mM Na dithionite
. the same, oxidised with 2 mM Na persulphate

Fig. 3.3.12. Spectra of control preparation of F_{3a} in

. Tris-HCl buffer, pH 7.0
. the same, reduced with 1 mM Na dithionite
. the same, oxidised with 1 mM Na persulphate
. difference spectrum of the reduced and oxidised protein

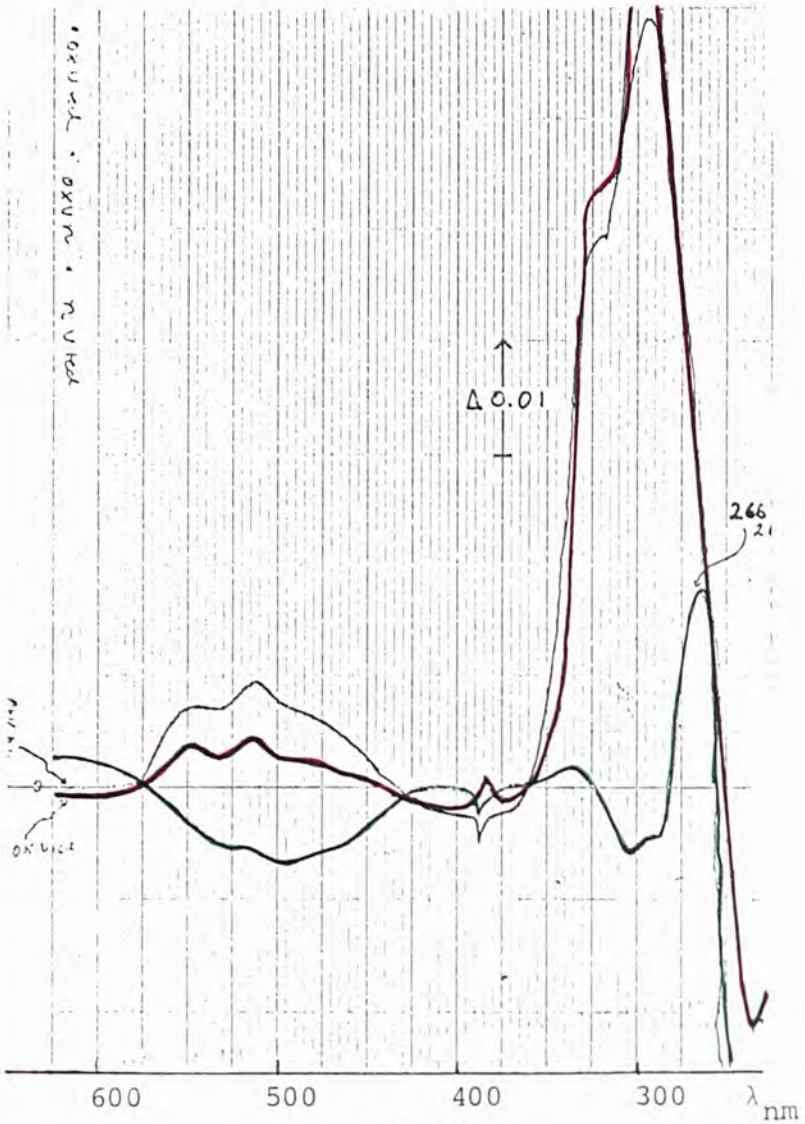


Fig. 3.3.13.b

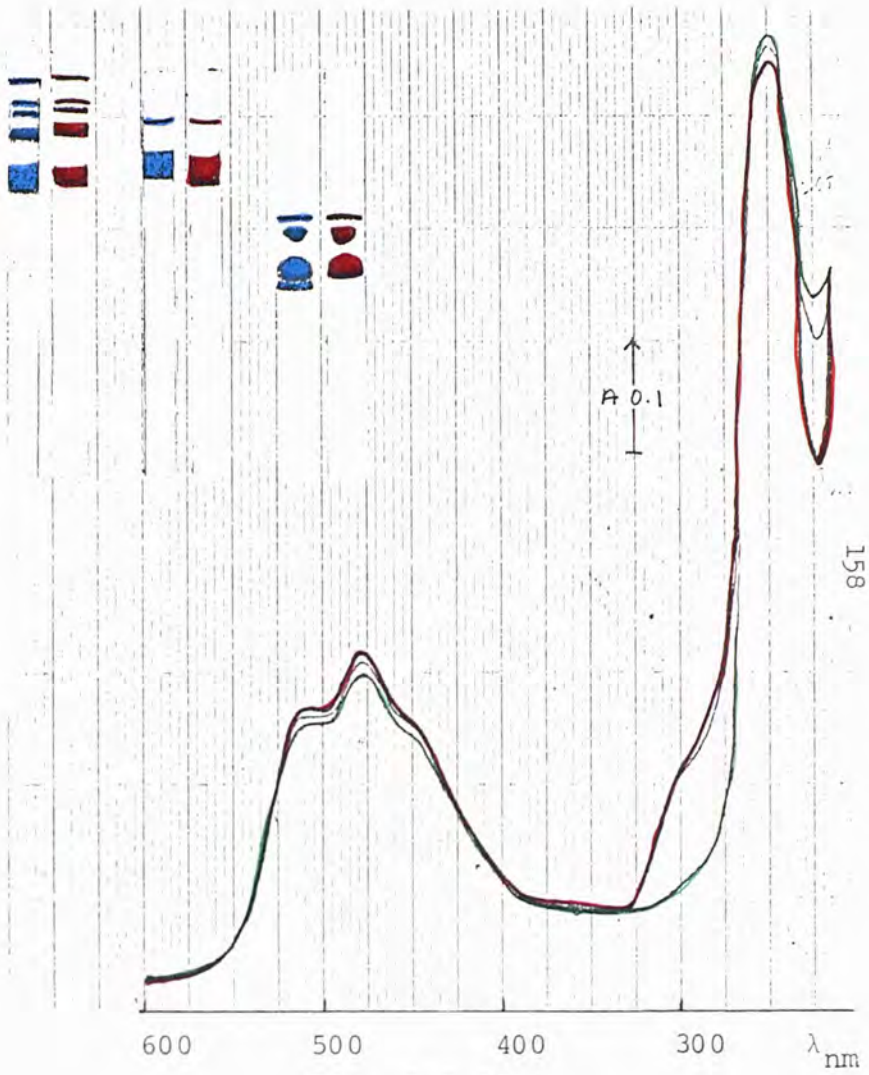


Fig. 3.3.13.a

Fig. 3.3.13.a Electrophoretic and spectral characteristics of the fraction of highest mobility at pH 8.3, F_{4a}, dissolved in 0.1 M Tris-HCl buffer, pH 7.0 (control)

- reduced (1 mM Na dithionite). $E_{280/510} = 2.7$
- oxidised (1 mM Na persulphate)

and

Fig. 3.3.13. b shows the corresponding difference spectra

- reduced against oxidised protein
- control against reduced protein
- control against oxidised protein

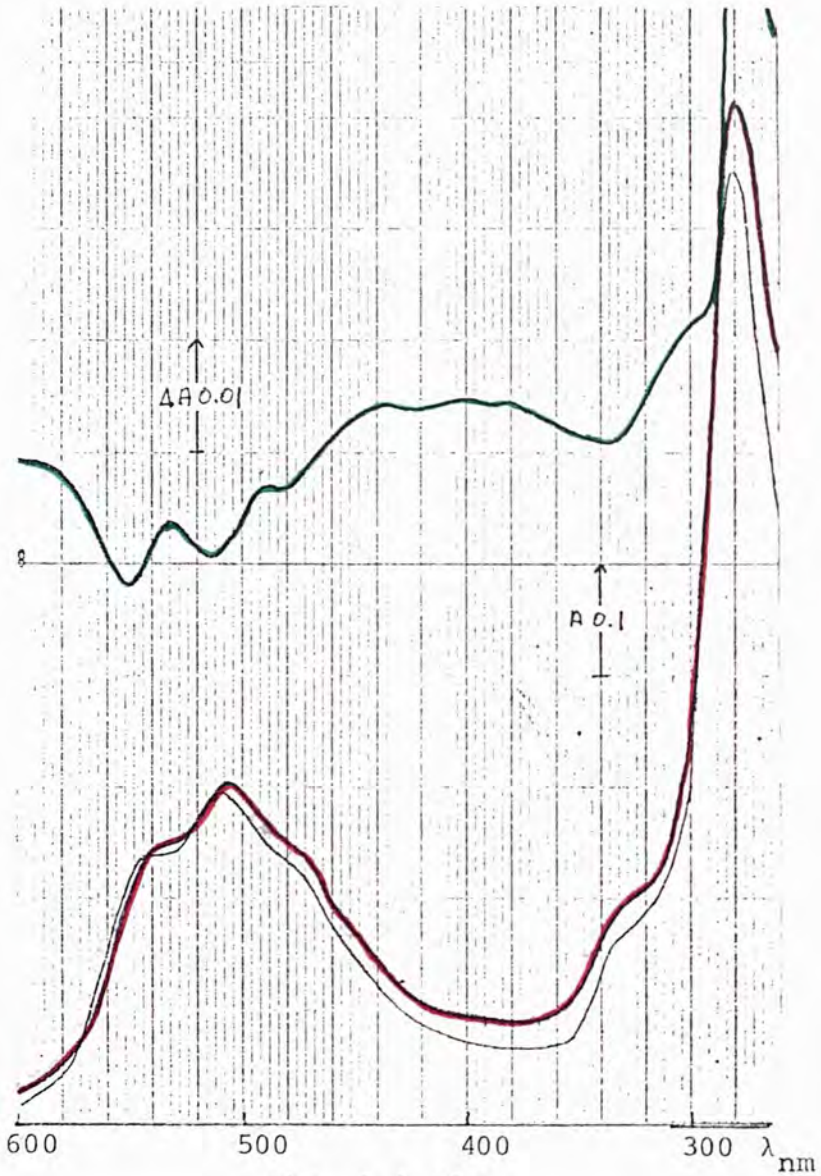


Fig. 3.3.13.c

Fig. 3.3.13.c CO-binding properties of F_{4a}
. control in 0.1 M Tris-HCl buffer, pH 7.0
. CO-treated F_{4a} after 2 min. exposure to CO
. difference spectrum of the CO-treated protein against the control sample

The CO-binding properties of fraction F_{1a} to F_{4a} show essentially identical patterns.

3.3.1.e Re-electrophoresis of fractions obtained by micro-preparative electrophoresis at pH 8.3 and 4.5

Re-electrophoresis of the fractions obtained by micropreparative electrophoresis shows that each purified fraction gives rise to the seven original fractions (see electrophoretograms on Fig. 3.3.7.a, 8, 9.a, 10, 11, 12 and 13.a).

3.3.1.f Subunit pattern of ovorubin, its apoprotein, prepared on Con A-Sepharose or by the method of Cheesman, and its electrophoretically prepared subfractions on SDS-PAGE

Investigation by SDS-PAGE in order to determine whether ovorubin and its electrophoretically prepared subfractions display differences in subunit structure, show that all electrophoretically prepared fractions are identical except for F_{oa} which, like the original Con A-purified protein, still contains some fractions of low mobility. All sub-units, except for the one of highest mobility, show a positive PAS reaction for protein-bound carbohydrate.

Experiments were carried out as described under 3.2.9.

Because of the uncertainty of molecular weight determination for glycoproteins, it was decided to determine the two molecular parameters, i.e. M_o and K_R, that together largely govern the migration behaviour in any electrophoretic system and compare them with standard protein polymers.

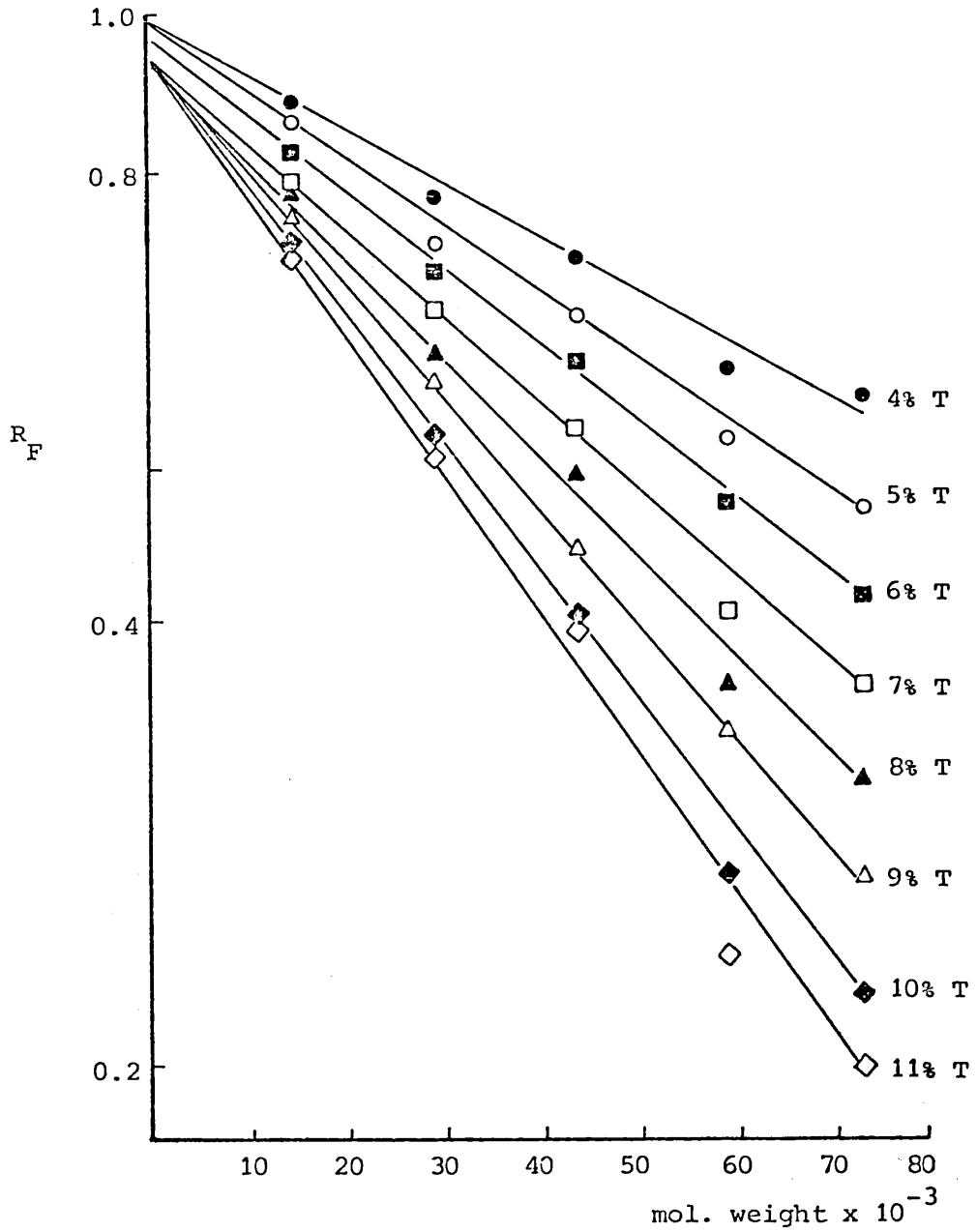

1.0
0.8
0.4
0.2
R_F
4% T
5% T
6% T
7% T
8% T
9% T
10% T
11% T
10
20
30
40
50
60
70
80
mol. weight x 10^{-3}

Fig. 3.3.14.

Fig. 3.3.14. shows that a plot of log R_F versus molecular weight is linear for the protein standards employed with gel concentrations between 4% and 11%T.

With the buffer system used, it was found that at 8% acrylamide concentration, bromophenol blue and pyronin Y were of equal mobility. Thus, for reasons explained earlier, at gel concentrations between 4 and 8%T R_F values are calculated relative to pyronin Y; at higher gel concentrations, bromophenol blue is taken as the tracker dye.

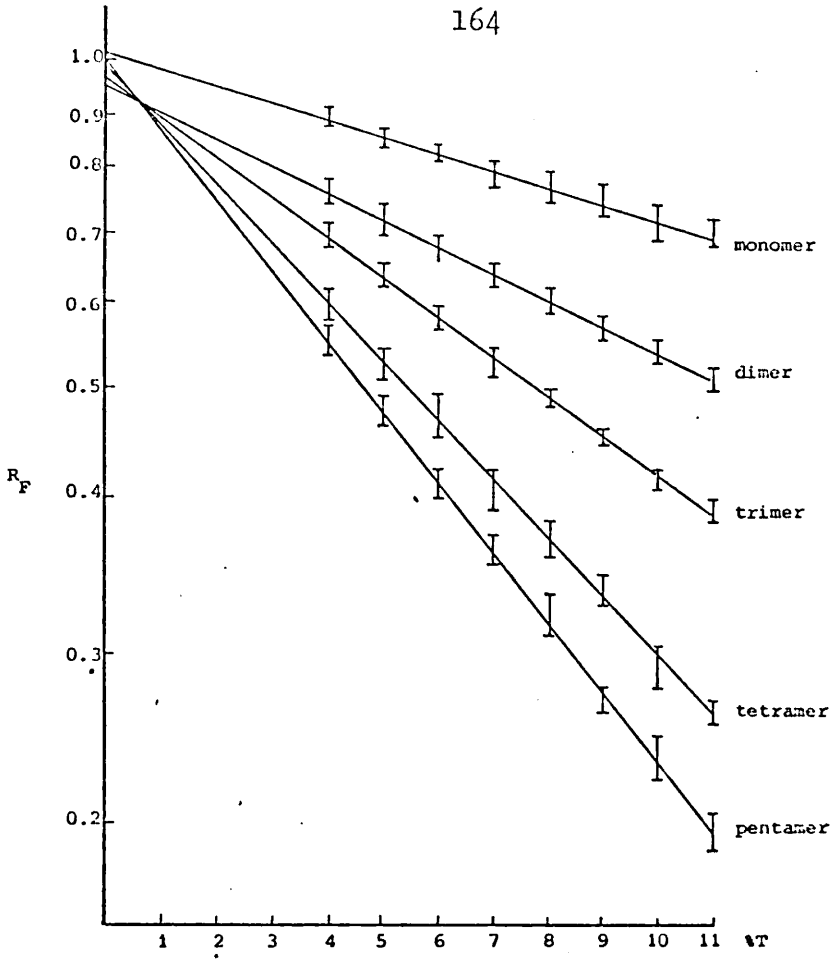


Fig. 3.3.15.a

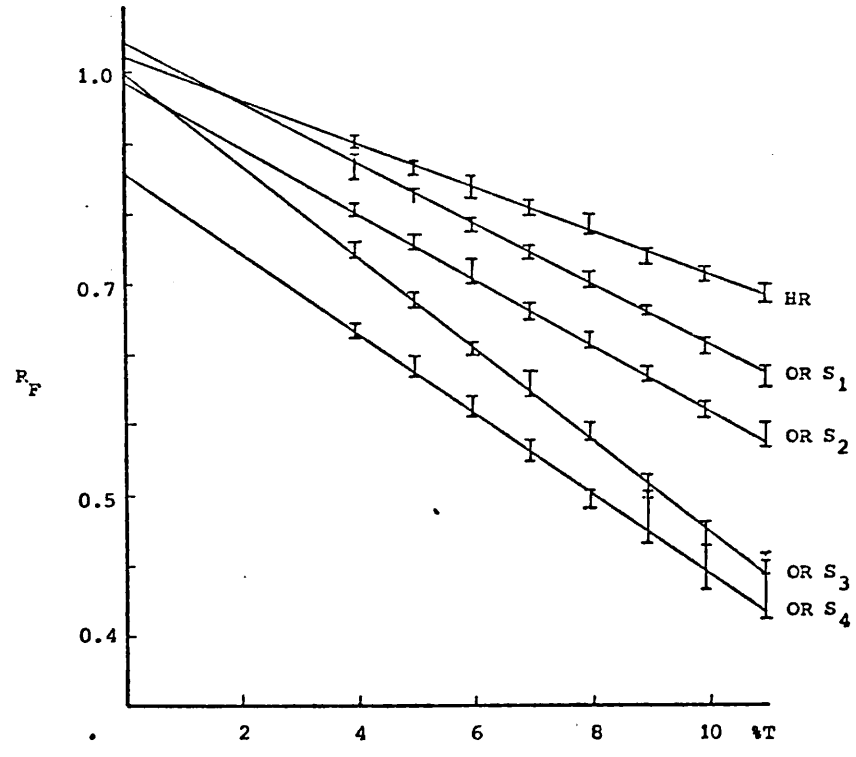
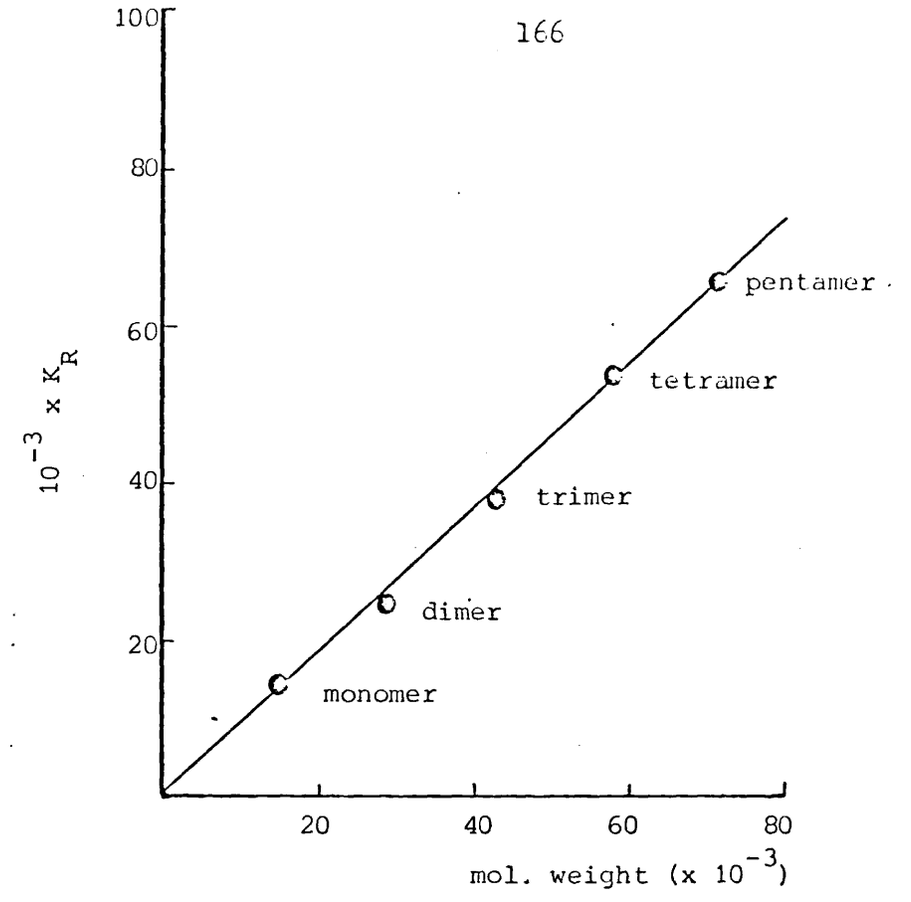


Fig. 3.3.15.b

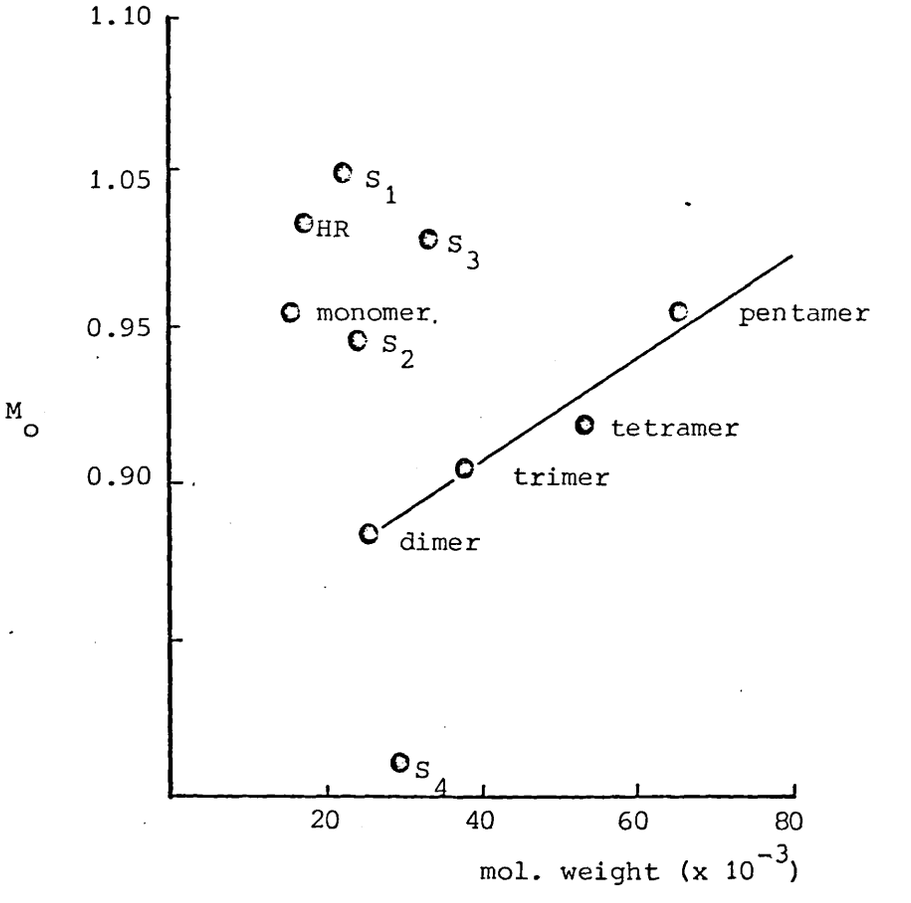
Fig. 3.3.15.a and b Typical "Ferguson Plots" for the standard protein polymers and for helicorubin P (HR) and the sub-units of ovorubin (OR)

From the intercept at T = 0, log M_o, and thus the free electrophoretic mobility, is obtained, while the retardation coefficient, K_R, is determined from the slope.



The relationship between mol. weight and K_R

Fig. 3.3.16.



The relationship between mol. weight and M_o

Fig. 3.3.17.

polypeptide	R_F at %T								10^{-3} x apparent mol. weight obtained from R_F at %T								10^3 x K_R	M_o	95% confidence limits for		Molecular weight obtained from K_R	PAS reaction
	4	5	6	7	8	9	10	11	4	5	6	7	8	9	10	11			K_R	M_o		
ovorubin S_1	0.86	0.82	0.785	0.75	0.711	0.68	0.645	0.61	17.5	18	18.5	18.5	18.5	18.5	19	19.5	21.27	1.05	21.07-21.47	1.047-1.055	23.700	neg
ovorubin S_2	0.8	0.76	0.72	0.68	0.65	0.61	0.58	0.55	25	25	25	24.5	24.5	24.5	24	23.5	23.29	0.992	23.04-23.53	0.988-0.996	26.100	pos
ovorubin S_3	0.745	0.69	0.64	0.596	0.553	0.51	0.49	0.44	35	34.5	34.5	34	34	34	33.5	33	32.37	1.005	32.065-32.67	0.999-1.010	36.100	pos
ovorubin S_4	0.66	0.62	0.58	0.54	0.505	0.47	0.44	0.414	48	45	43.5	41	40.5	40	37.5	37	28.77	0.858	28.45-29.09	0.853-0.863	32.300	pos
helicorubin	0.895	0.86	0.83	0.8	0.77	0.745	0.72	0.69	13	13.5	14	13	12.5	14.5	13.5	13	15.37	1.026	15.19-15.54	1.023-1.029	17.200	neg

Table 3.3.4.

Molecular characteristics of the subunits of ovorubin obtained in SDS-PAGE electrophoresis

Values for K_R and M_o were obtained as described under 3.3. methods and materials.

95% confidence limits for K_R and M_o were computed by the method of Rodbard and Chrambach (1974).

Table 3.3.4. gives the apparent molecular weights at T values from 4-11%, free electrophoretic mobilities and retardation coefficients, the 95% confidence limits thereof, molecular weights determined from the retardation coefficients and the staining characteristics of helicorubin and the subunits of ovorubin. The R_F values given for the molecular weights of subunits are the pooled mean values from series of 8-12 determinations for ovorubin purified on Con A-Sepharose, on calcium phosphate gel, and for the apoprotein prepared by both methods and 4-6 determinations for each fraction of ovorubin purified by micropreparative electrophoresis at each %T.

The free electrophoretic mobilities of the standard protein polymer-SDS complexes are very similar. However, in the molecular weight range between 28,600 and 71,500, there is a small (7.5%) but linear increase in mobility with increasing molecular weight. This is demonstrated in Fig. 3.3.17. where a linear relationship is shown between the retardation coefficient K_R, (which is proportional to molecular weight, see Fig. 3.3.16.) and M_o. Over this range, the effective size of protein-SDS complexes hence increases linearly with increasing molecular weight of the protein, and this is accompanied by a slight, but significant increase in M_o. In the molecular weight range below 25,000, no linear relationship is obtained for K_R and M_o, an observation also made by other workers (Banker and Cotman, 1972, Frank and Rodbard, 1975).

3.3.1.g E.s.r. spectrum of ovorubin

The e.s.r. spectrum of ovorubin, recorded at room temperature by Dr K. Sales shows strong signals at g = 1.96 and 4.2.

Elementary analysis for iron and copper performed during the early stages of this investigation on ovorubin prepared by the method of Cheesman suggested the presence of approx. 0.6 moles of both iron and copper per mole of ovorubin.

3.3.2. Purification of helicorubin

It soon became apparent that helicorubin from *P. canaliculata*, in spite of having similar spectral characteristics to those of the helicorubin from *Helix pomatia* (Keilin 1956, 1957, 1968)

is a different protein. It cannot be absorbed, like the *Helix* protein, on DEAE cellulose (DE 32). On dialysis against distilled water in a dialysis membrane of 24 Å pore size, it is adsorbed on the membrane, a property shared with cytochome *c* from horse heart or *Candida krusei*.

The purification scheme for helicorubin from *P. canaliculata* (helicorubin P) is outlined in Fig. 3.2.2.

Helicorubin P, unlike Keilin's helicorubin, is a basic protein. The purified haemoprotein is electrophoretically homogeneous in a β-alanine-acetic acid buffer at pH 4.5 SDS-PAGE ranging from T=4-11% gives values for the molecular weight between 12,500 and 14,500 as calculated from R_F, while the value obtained from the retardation coefficient is 17,500. On Sephadex G75 and G200, values of 15,500 and 16,750 are obtained.

	α-band		β-band		γ-band		δ-band		$\frac{E_{275}}{E_{428}}$
	λ (nm)	ε_{mM}	λ (nm)	ε_{mM}	λ (nm)	ε_{mM}	λ (nm)	ε_{mM}	
reduced	559.5	30	529	17	428	147	334	33	0.36
oxidised	-	-	-	-	416	91	-	-	0.29

Table 3.3.5.

Spectral characteristics of helicorubin P. ε_{mM} values are based on a mean molecular weight of 15,200 obtained from pooled electrophoretic and chromatographic determinations.

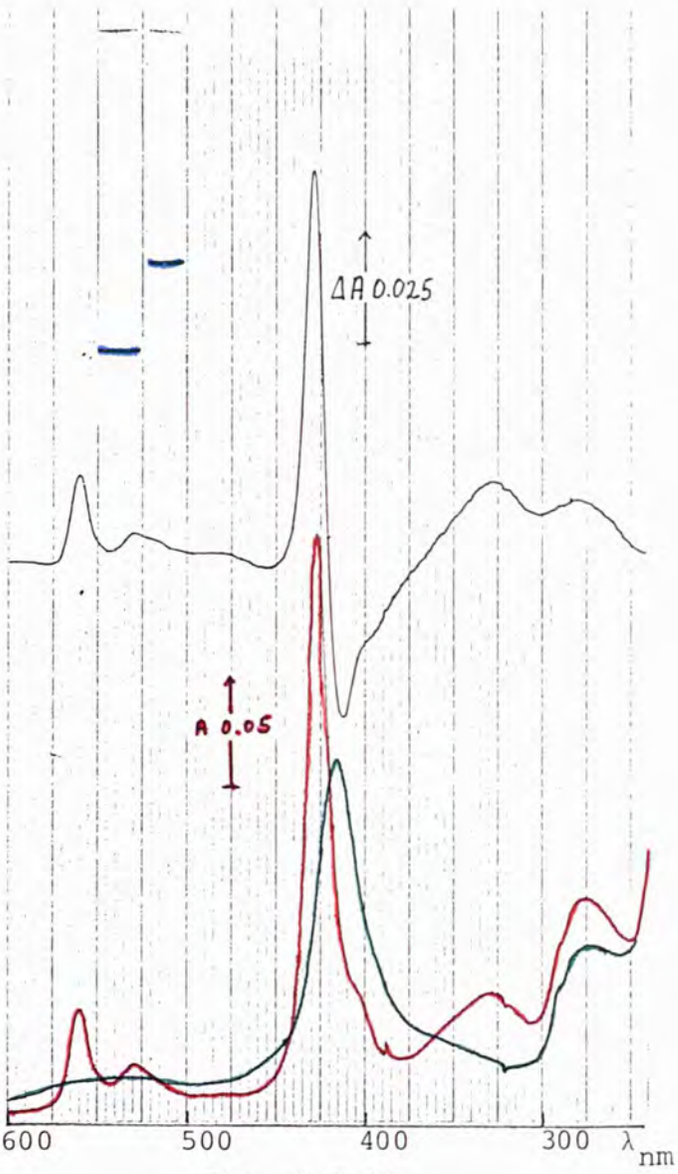


Fig. 3.3.18.

Fig. 3.3.18. Electrophoretic and spectral characteristics of helicorubin P, purified as outlined in Fig. 3.2.2. dissolved in 0.1 M Tris-HCl buffer, pH 7.0

- 0.065 mg ml^{-1} helicorubin reduced by 1 mM Na dithionite
- the same, oxidised with 1 mM Na persulphate
- the difference spectrum thereof, with the reduced protein in the test cell and the oxidised protein in the reference cell (path length 0.4375 cm)

Electrophoretic pattern from left to right

- at pH 4.5 in a β-alanine-acetic acid buffer system
- SDS-PAGE at pH 8.3 in a Tris-HCl buffer system

The spectrum of helicorubin P is not affected by CO.

For experimental details see 3.2.4., 5, 6 and 9.

3.4. Discussion

3.4.1. The molecular characteristics of ovorubin

Comparison of spectra of ovorubin prepared by the method of Cheesman (Fig. 3.3.4.b) and that obtained by adsorption on Con A-Sepharose and elution with α-methylglucose show that the latter method gives lower $E_{280/510}$ ratios. (Fig. 3.3.4.a). This is consistently found, as can be seen from Table 3.3.1. which shows that Con A-purified ovorubin invariably has a lower $E_{280/510}$ ratio. The reason for this has been traced to a contamination of ovorubin prepared by the method of Cheesman by a high molecular weight polysaccharide co-purifying with the carotenoglycoprotein on calcium phosphate gels. Whether this contamination is due to a blood group A-like antigenic substance found in the eggs of *P. canaliculata* (Uhlenbruck *et al*, 1976) requires immunological investigation.

This polymer, which appears to be insoluble at low ionic strength, seems to have a high affinity for ovorubin, since the latter coprecipitates with it at low ionic strength. There is also suggestive evidence that this substance in some way protects the chromophore of ovorubin absorbing at 330 nm from oxidising and reducing agents, since ovorubin prepared by the method of Cheesman is far less susceptible to such chemicals (see Fig. 3.3.4.a and b).

The fact that ovorubin purified by the method of Cheesman invariably shows an $E_{280/510}$ ratio of 2.6-2.8 suggests that a constant amount of the polysaccharide co-purifies with it.

Although there cannot be any doubt that ovorubin is a glycoprotein, the carbohydrate analysis by Norden (1962, 1972) may be questionable in view of the finding that ovorubin is accompanied on CaP gel by a material of high molecular weight giving a positive Molisch test. Evidence obtained during the present investigation clearly indicates that the chromophore absorbing in the 330 nm region is not associated with the carotenoid moiety, but is an integral part of the apoprotein. The previous suggestion by Cheesman

(1958) that this feature was due to the carotenoid moiety may have arisen from the fact that his absorption spectra for the apo-ovorubin were recorded on aged preparations (Cheesman, personal communication).

Preliminary investigations by the method of Velick *et al.* (1957) provide no evidence that this chromophore may be a perturbed NAD(P)H molecule firmly bound to ovorubin. The identity of this chromophore, which is light-sensitive and disappears on ageing, requires further investigation. At present it appears likely that a non-haem iron prosthetic group may account for this spectral feature. Iron-sulphur proteins are commonly associated with yellowish-brown pigmentation and evidence for such a prosthetic group comes from e.s.r. spectra of ovorubin which show strong signals at 1.96 and 4.2 g. The signal at 1.96 g is characteristic of nitrogenases which contain both iron and molybdenum (Hardy and Burns, 1973). Preliminary investigations have shown that ovorubin purified by the method of Cheesman contains 0.6 moles of Fe and Cu per mol of protein. This low value can probably be accounted for by the fact that ovorubin purified on CaP gel is impure.

At the present stage of the investigation it is premature to speculate about a function of ovorubin in nitrogen fixation solely on the basis of its e.s.r. spectrum. A substantial amount of the absorption from 260-280 nm is contributed by the iron-sulphur group(s) in nitrogenases (Hardy and Burns, 1973). The spectrum of ovorubin in the region is certainly sensitive to oxidation and reduction (See Fig. 3.3.1.b, 2.b or 3.3.3.b) and it is conceivable that these changes reflect a variation in the level of oxidation of the non-haem iron prosthetic group. The CO-binding properties of ovorubin appear in some way to be associated with the carotenoid moiety, since the apoprotein displays no spectral changes on exposure to this gas. Difference spectra of the apoprotein in the reduced and oxidised state are virtually indistinguishable from that of ovorubin except in the region of carotenoid absorption. It is therefore unlikely that during the preparation of the

apoprotein a chromophore such as a quinone is removed from the protein, which could also account for the e.s.r. signal. In view of the CO-binding properties of ovorubin, it is tempting to speculate that the carotenoid moiety has a function in O_2 binding especially since O_2 and CO binding involve identical functional groups. Such a role for carotenoids in invertebrates has been proposed by Karnaukhov (1971). An analogous conclusion does not seem fully justified here, since the carotenoid moiety, if not involved in CO-binding, may possibly confer a tertiary conformation on the ovorubin molecule which allows for CO binding; this in turn may lead to a slight change in the mode of binding of the carotenoid.

The light-lability of the CO-ovorubin complex (see Fig. 3.3.1.d) is a feature characteristic of CO-binding haemoproteins.

The observation that the fraction of ovorubin eluted with borate from Con A-Sepharose has a consistently higher $E_{280/510}$ than ovorubin eluted from the lectin with α-methyl-glucose, suggests that the apoprotein has a higher affinity for the adsorbent than the carotenoglycoprotein; the contaminating polysaccharide, which contributes to the absorbance at 280 nm, appears to be completely removed from ovorubin after thorough washing of the column with M NaCl. Electrophoretic analysis, furthermore, shows no evidence of an additional protein in the borate-eluted fraction. The difference spectrum for the reduced and oxidised forms of this fraction is, however, unusually accentuated in the 330-310 nm region; the significance of this requires further investigation.

Electrophoresis on gradient gels show that at pH 8.3 five bands are obtained. They correspond approximately to forms of the protein from monomeric to pentameric. This is supported by the observation that, after micropreparative electrophoresis, identical subunit patterns on SDS-PAGE are obtained with all fractions except that remaining near the origin at pH 8.3.

The fact that the molecular weights of fractions obtained by electrophoresis on gradient gels are not exact multiples

of the apparent monomer is most certainly due to the fact that with all molecular weight determination techniques based on physical size, the shape of the molecule affects migration. The anomalous migration behaviour of glycoproteins in comparison to standard proteins devoid of the carbohydrate prosthetic groups is a well-known phenomenon.

A rather surprising finding is that the apoprotein of ovorubin shows an electrophoretic pattern identical with that of the holoprotein not only when separation is based on molecular weight, i.e. on gradient gels, but also when fractionation is carried out on the basis of charge characteristics. The carotenoid evidently does not influence the zeta potential of the protein; it may thus be buried within the molecule.

Electrophoretic separation of the cathodic fractions at pH 4.5 by both charge and molecular weight characteristics give rise to two fractions which appear to constitute a monomer (F_{1c}) and dimer (F_{2c}) of ovorubin (Table 3.3.2.).

Again, the subunit patterns on SDS-PAGE obtained after micropreparative electrophoresis of these fractions show that they are identical, and there is no difference in subunit pattern on comparison with the anodic fractions $F_{1a} - F_{4a}$.

A possible explanation for the finding that only monomeric and dimeric fractions are obtained on electrophoresis at pH 4.5 is that, at this pH, the configuration of the monomer is such as not to favour aggregation.

The spectral characteristics of ovorubin purified by micropreparative electrophoresis vary considerably. Spectra of the cathodic fractions of ovorubin show little evidence of the chromophore in the 330 nm region and the spectra of the CO-complexes of these fractions are distinctly different from those of the original ovorubin. It seems likely that the tertiary structure of the protein has undergone a change which influences the mode of binding of CO to the protein (see Fig. 3.3.7.c and a).

When the fractions are left for long periods in Tris-HCl

buffer at pH 8.3, a slow reversion to the initial conformation appears to take place, since after 20 hrs the spectrum of the CO-complex approaches that of ovorubin and its anodic fractions.

Cold-denaturation and heat-promoted renaturation provide further evidence that at pH 4.5 a change in the tertiary structure of protein occurs which renders it predominantly dependent upon hydrophobic interactions. The consequent denaturation at low temperatures is attended by the observed hypsochromic shift of some 85 nm.

It is impossible as yet to decide whether the disappearance of the chromophore absorbing at 330 nm is brought about by the pH of the buffer or is due to ageing of the protein during the long purification procedure. Acid-labile sulphur has been observed with ferredoxins of either plant or bacterial origins (Beinert, 1973). Ageing is most certainly the reason for the disappearance of this spectral feature in anodic fractions. This is shown by the observation that F_{4a}, which is quantitatively the most important anodic fraction and can be obtained in a pure form in adequate quantities for experimentation within 10 days, still shows a distinct shoulder in the 330 nm region.

Fractions F_{3a} to F_{1a} show decreasing absorbances in this region. However, these fractions are also quantitively less important, and therefore the time taken to obtain sufficient quantities for experimentation varies from 10 (F_{4a}) to 25 days (F_{1a}, F_{1c}, F_{oa}), with intermediate periods of 13 to 20 days for F_{2a}, F_{3a} and F_{2c}.

Caution must hence be observed in interpreting the spectra of these fractions, which are most certainly in an advanced state of denaturation.

The fraction remaining at the origin at pH 8.3 in a 4% gel quantitatively co-purifies with the contaminating polysaccharide. Separation can be achieved by chromatography on Sephadex G200 or Con A-Sepharose. It is likely that this substance confers upon the protein its low solubility at low ionic strength.

On re-electrophoresis, each fraction obtained by micro-preparative electrophoresis gives rise to the original seven fractions. Quantitative differences are, however, obtained in that each fraction on re-electrophoresis shows a relative increase in the amount of the fraction from which it was derived. The important fact, however, is that all fractions are interconvertible, clearly indicating that we are concerned with a single protein as, indeed , is suggested by the identity of the subunit patterns in SDS-PAGE.

A separation pattern similar to that observed for the anodic fractions is found with chromatographic separation on Sephadex G200, where five fractions were obtained which seemed to represent the monomer and aggregates up to the pentamer, with the monomeric form predominating, a pattern already seen with the electrophoretic separation.

The increase in the $E_{280/510}$ ratio accompanying fractions of lower elution volume may be taken as suggestive evidence that the apoprotein contribution in these polymeric fractions is quantitatively of greater importance.

Ovorubin has four different subunits on SDS-PAGE, three of which contain bound carbohydrate. When compared with standard proteins, molecular weight determinations of carbohydrate-containing polypeptides commonly give rise to erroneous values. Results shown in Table 3.3.4. show that molecular weights obtained from measurements of relative electrophoretic mobility at different values of T are in fairly good agreement with those obtained from K_R measurements for ovorubin subunits designated S_1 to S_3. The fourth subunit, however, shows a marked decrease in electrophoretic mobility, and it can be shown that there is an increase in apparent molecular weight as %T decreases. The molecular weight obtained from the retardation coefficient is nearly one third lower than that obtained from its relative electrophoretic mobility on a 4% gel. This clearly indicates that the molecular weight obtained for this subunit in gels at a single T is invalid. The anomalous migration of this subunit is presumptive evidence that one

or both of the two conditions for the validity of molecular weight determination by this method have not been met. The first condition is that the protein under investigation must bind the same amount of SDS on a gram for gram basis as the standards, and thus have the same charge density and free electrophoretic mobility; the second condition is that the relationship between hydrodynamic size, usually expressed as the Stokes radius, and molecular weight must be identical for both unknown and standards. However, since M_o and K_R can be independent variables (Banker and Cotman, 1972) it is possible to have anomalous behaviour of a protein in SDS-PAGE suchthat M_o differs considerably from that of the standards, while K_R has the appropriate relationship to molecular weight. Whether this applies in the present case cannot be determined without additional physicochemical information.

Use of K_R in the molecular weight determination seems to be justified for the three subunits of ovorubin with the highest mobilities.

Although there appear to be definite advantages in determining molecular weights for protein by SDS-PAGE using the relationship between K_R and molecular weight rather than between log R_F and molecular weight at a single T, all molecular weight determinations of glycoproteins must be viewed with reserve, especially when anomalous behaviour is revealed (Banker and Cotman, 1972; Rodbard and Chrambach, 1974). The molecular weight for the apparent subunit of ovorubin of lowest mobility was not calculated, since its value of 150,000 or more is quite outside the range of reliable standards. This polypeptide is only found in ovorubin prepared on Con A-Sepharose or CaP gel without further purification, and is associated with the F_{oa} fraction from electrophoresis. It does not appear in any other fraction, and the obvious question arises, whether it is an integral part of the ovorubin molecule.

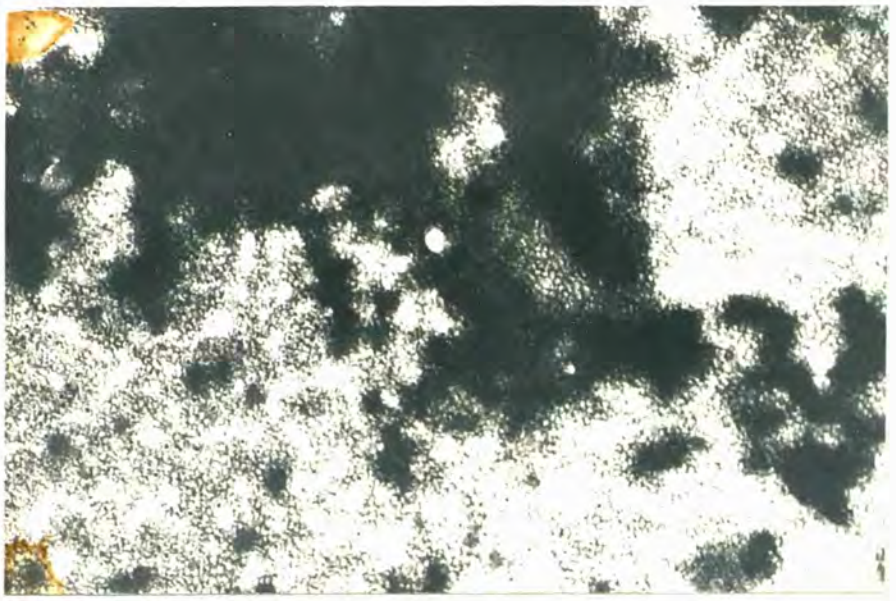


Fig. 3.4.1a
Electron micrograph of ovorubin prepared by affinity chromatography on Con A-Sepharose.
Magnification: 110,000

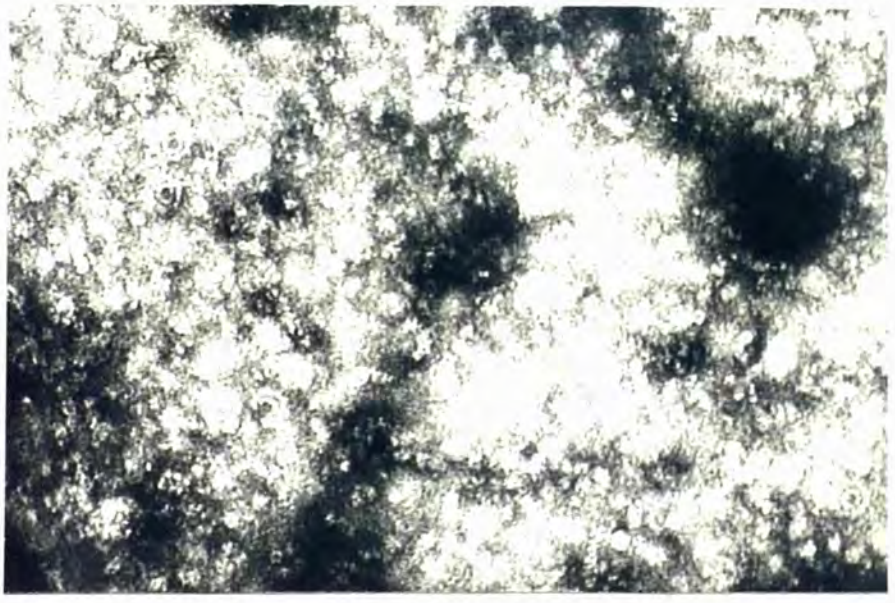


Fig. 3.4.1b
Electron micrograph of ovorubin prepared by the method of Cheesman.
Magnification: 180,000

It is certainly not involved in carotenoid binding, since all the fractions that are devoid of it still show the characteristic carotenoid peaks. Experimental evidence suggests (see Chapter 4) that it is this subunit that confers a phosphatase activity on ovorubin prepared on Con A-Sepharose or calcium phosphate gel.

A preliminary anlaysis by densitometric scanning of the subunit pattern obtained on 5% SDS-PAGE gives a result consistent with the subunit composition of ovorubin shown in Table 3.4.1.

Ovorubin sub-unit	M.W. x 10^{-3}	% from scanning	Probable no. of sub-units per molecule	% from M.W.	contribution to M.W.
S_1	23.7	20.5	3	21.6	71,100
S_2	26.1	52	6	47.7	156,600
S_3	36.1	9.5	1	11	36,100
S_4	32.3	18	2	19.7	64,600

Table 3.4.1.

Sub-unit composition of ovorubin based on analysis on SDS-PAGE

The distribution of sub-units deduced from SDS-PAGE experiments has been considered in the light of some electron micrographs of ovorubin molecules made by Mr. Rayner Jones of the Electron Microscope Unit at Bedford College (see Fig. 3.4.1.a and b).

Preparations of ovorubin made by adsorption on Con A-Sepharose (Fig. 3.4.1.a) and calcium phosphate (Fig. 3.4.1.b) have very similar appearances, although the former tend to show a higher frequency of the entities which are presumably the quaternary structures of ovorubin.

The ovorubin molecule appears to have most of its sub-units arranged in two concentric hexagons with two or three sub-units in a central position. The diameter of the structure is in the region of 19 nm. It is difficult to distinguish the numbers of globular particles contributing to each of the hexagonal structures. The number of such particles certainly exceeds twelve, the number of sub-units

determined in SDS-PAGE. It must be concluded that many of these structures are paired in the sub-units.

Cheesman (personal communication) has proposed the following: If it be assumed that the body observed in EM is the ovorubin molecule of molecular weight in the region of 330,000, and the structure is a compact one, a simple calculation, based on a partial specific volume = 0.75, gives a mean thickness of 1.7 nm. Sub-unit S_3, having an apparent molecular weight of 36,100, is accommodated in the centre of this molecule and, on considerations of symmetry, may be involved in the binding of the carotenoid moiety. Adjacent to S_3 are two sub-units of S_4. This central complex is thought to be surrounded by S_1 sub-units. It is suggested that these have a dumb-bell shape, appearing as pairs of globules in the electron micrographs. Three such pairs may constitute the inner hexagon. Similarly dumb-bell-shaped sub-units, six in number, form the outer hexagon. These represent the S_2 fraction in SDS-PAGE.

It is proposed that the values shown in Table 3.4.2. can accommodate both the observation from electron microscopy (E.M.) and the sub-unit molecular weight values obtained on SDS-PAGE.

Subunit	MW $\times 10^{-3}$	molecular radius Å	No. of sub-units	Total cross-sectional area Å²	Vol Å³
S_1	23.7	15.2	6 x ½	4360	88300
S_2	26.1	15.75	12 x ½	9350	196400
S_3	36.1	22.1	1	1530	45200
S_4	32.3	21.3	2	2850	81000

Table 3.4.2.

Relationship between sub-unit molecular weight obtained on SDS-PAGE and molecular pattern obtained on electron micrographs

Calculations of molecular radius and cross-sectional area are made on assumption that the particles as seen in electron micrographs are spherical and that sub-units S_1 and S_2 are each made up of two identical spheres. Molecular dimensions

of ovorubin, i.e. molecular area, thickness and volume, when calculated on this basis, show good agreement with measurements obtained from electron micrographs as shown in Fig. 3.4.1.a and b.

	Basis of calculation	
molecular area $Å^2$	Cross-sectional area of sub-units	2.3×10^4
	E.M. measurements	2.3×10^4
molecular thickness Å	Mean radii of sub-units	17.2
	E.M. & Mol. Wt.	17.5
molecular volume $Å^3$	Dimensions of sub-units	4.0×10^5
	E.M. & Mol. Wt.	4.0×10^5

Table 3.4.3.

Molecular dimensions of ovorubin calculated from experimental data.

The calculation of molecular area based on the sub-units was made by multiplying the summed cross-sectional areas of the units by a factor $\frac{4}{\pi}$ which gives a correction for dead space between rigid spheres in a square lattice. The corresponding molecular thickness is calculated from the mean molecular radius by multiplying by the factor $\frac{\pi}{3}$. The molecular volumes represent the products of the respective values for molecular area and thickness.

If the molecular model described above were correct, it would suggest that there is a very strong tendency for the sub-units S_1 and S_2 to form an aggregated state owing to strong intermolecular forces. These forces might stabilise a self-aggregated state of lower free energy. A more adequate sub-unit analysis may possibly be achieved by increasing the mercaptoethanol and SDS concentrations employed in SDS-PAGE. Under such conditions, structures such as the dumb-bells proposed above might be dissociated into smaller units.

3.4.2. Helicorubin P

Molecular weight determinations for helicorubin P show considerable variations, i.e. a spread from 12,500 at 8%T to 17,200 when calculated from K_R. Intermediate values are obtained from gel filtration experiments. Non-standard migration on SDS-PAGE for basic proteins like histones has frequently been observed. Since helicorubin P, in contrast to helicorubin from *Helix pomatia*, is a basic protein, the observed variation in molecular weight may possibly be accounted for by the amino acid composition of this protein. (Preliminary data obtained from isoelectric focusing of helicorubin P performed by a Pharmacia representative indicate that the isoelectric point is in the region of pH 9.8).

Since all values for molecular weight determinations are higher than that obtained by Keilin (1968), it is likely that a chain rich in basic amino acids may have been added to the protein. This does not appear to influence the spectral characteristics of the haemoprotein in any significant way, since the position of the absorption maxima and the ratio of the reduced and oxidised Soret bands are virtually identical. Although the ε_{mM} for helicorubin P based on an weighted mean molecular weight is *ca.* 15% lower than that of helicorubin from *Helix pomatia*, it is interesting to note that almost identical values would be obtained for the two proteins if calculations were based on the molecular weight obtained from K_R, i.e. 17,200. The difference between these two proteins is interesting from an evolutionary point of view.

CHAPTER 4

SOME PROPERTIES OF AN ATP-DEPHOSPHORYLATING ENZYME PRESENT IN EGG HOMOGENATES OF *POMACEA CANALICULATA*

4.1. Introduction

In recent years evidence has been accumulating that the energy-transducing and conserving mechanisms of a variety of tissues and organelles possess, at least in part, a similar transphorylating mechanism. A notable exception is the light-driven hydrogen ion pump coupled to ATP synthesis found in extremely halophile bacteria (Osterhelt and Stoeckenius, 1973). This transphosphorylating mechanism was traced to a protein component with a molecular weight in the region of 330 - 350,000. It can be removed from its place in the energy-transducing membranes of bacteria, mitochondria or chloroplasts without disturbing electron transport. However, phosphorylation of ADP by P_i during electron transport depends on the presence of this protein which has been called the coupling factor, since its return to depleted membranes can to some extent restore the link between electron transport and energy conservation (Pullman *et al.*, 1960, Penefsky *et al.* 1960, Pullman and Monroy, 1963).

The coupling factor displayed *in vitro* a highly active dinitrophenol-stimulated ATPase activity which was viewed as an artefact of isolation. When incorporated into an energy-transducing membrane, it was thought to function in the direction of ATP synthesis and to participate in the terminal transphosphorylating reaction (Penefsky *et al.* 1960).

This ATPase showed characteristic pH-activity curves. Mitochondria of rat liver and brain exhibit three maxima at pH 5.9-6.3, 7.0-7.5 and 8.3-8.5. (Myers and Slater, 1957 ; Holton *et al.*, 1957; Lagnado *et al.*, 1959)

Since some ATP synthesis has been observed in homogenates of *P. canaliculata* eggs, a search was made for a similar enzymic activity.

4.2. Materials and Methods

Egg homogenates, homogenates freed from ovorubin, and ovorubin and its subfractions were prepared as described in chapter 3.2 except that

in the present experiments the eggs were homogenised in ice-cold distilled water and the Con A-Sepharose was washed with this several times before use. All chemicals employed in the ATPase assay were obtained from BDH, except for ouabain (Sigma) and oligomycin (Boehringer). All reagents used were stored in a desiccator at 4°C. The concentrations of adenine nucleotides were determined from their molar absorbances at 260 nm.

Glassware used throughout was thoroughly cleaned in conc. chromic-sulphuric acid, followed by several washes in distilled water, 10% HCl., distilled and double distilled water to ensure complete removal of phosphate ions.

4.2.1 Determination of the adenine-nucleotide dephosphorylating activity.

Stock solutions were made up in distilled water at neutral pH, which was later adjusted by dilution with the appropriate buffer. All ATPase assays were carried out at 30°C. For the range between pH 5.2 and 8.0, a Tris-maleate buffer and for the range between pH 8.0 and 9.0 a Tris-HCl buffer system was used. The final concentration of buffer in the assay mixtures was kept at 0.1M. The ATPase activity was tested at intervals of 0.2 pH units.

The effects of $MgCl_2$, $CaCl_2$, NaCl, KCl (all at 10 mM final conc.) 10 µM ouabain in the presence and absence of Na^+ and K^+ ions, 0.1 µM oligomycin, 0.1 mM DNP (2 x recrystallised), 10% (v/v) methanol, 5 mM dithiothreitol, 5 mM Na dithionite, various concentrations of ADP (2.5-40 mM), exposure to 0.1% Triton X-100 and to 0.1% Na-deoxycholate at 30°C one hour before assay, freezing and thawing five times over a period of 10 hrs, as well as the effects of light (diffuse daylight) and darkness on the ATPase activity were studied.

Tubes were prepared containing 10 µl ATP solution with or without 10 µl of potential effector in 0.1M buffer, and the volume was adjusted with such an amount of 0.1 M buffer that subsequent addition of enzyme would produce a final volume of 100 µl.

Appropriate blank controls for reagents and enzyme were included in each experiment. All determinations were carried out in quadruplicate. The reaction was started on addition of the stock solution of either egg homogenate, homogenate freed of ovorubin, ovorubin or one of its subfractions to the reaction mixture, which had been preincubated at 30°C for 15 min. In cases where a reconstituted homogenate (i.e. a homogenate freed from ovorubin to which ovorubin purified in Con A-Sepharose had been added) was used, this was incubated for 30 min. at 30°C before the assay.

The reaction was stopped at 2.5, 5, 10, 15 or 30 min. by addition of an equal volume of 20% (w/v) ice-cold TCA. The contents were rapidly mixed, plunged immediately into ice and left for 15 min. before centrifugation at 10,000g for 30 min. at 4°C. Subsequently 0.1 ml aliquots were withdrawn for the determination of phosphate by the method of Ames (1966).

4.2.2 Analysis of the reaction products

The reaction products obtained in this way were analysed by chromatography on PEI (polyethyleneimine) ion-exchange paper after removal of TCA by shaking the mixture three times with a fivefold volume of ice-cold ether. The PEI paper was prepared by the method of Randerath (1963) as modified by Shirley (1978).

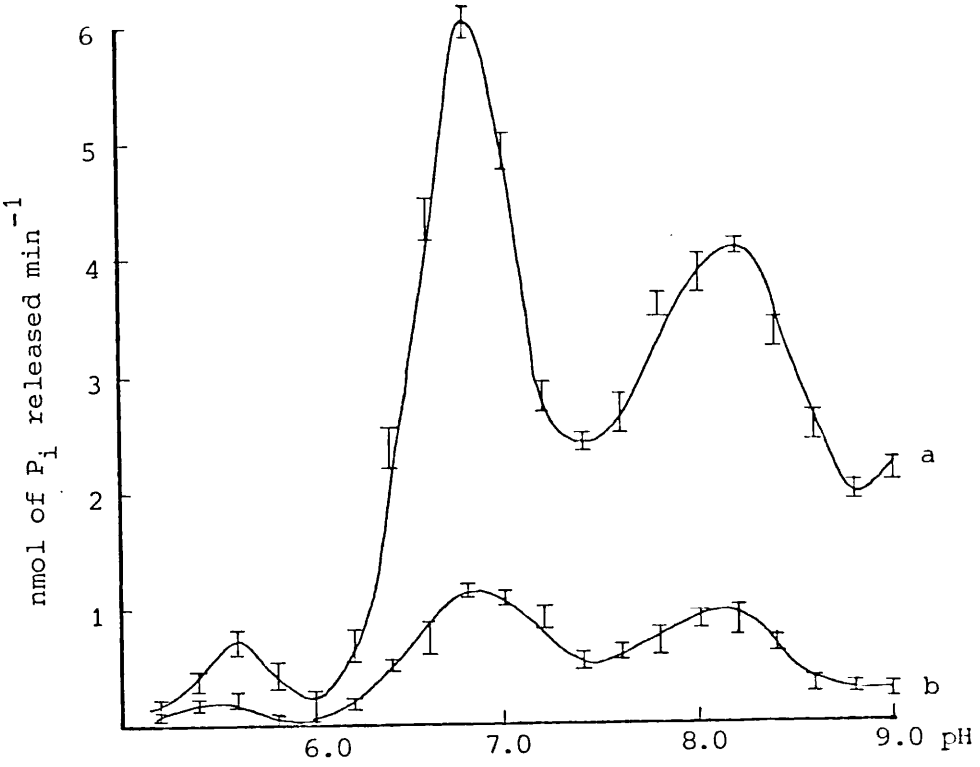


Fig. 4.3.1

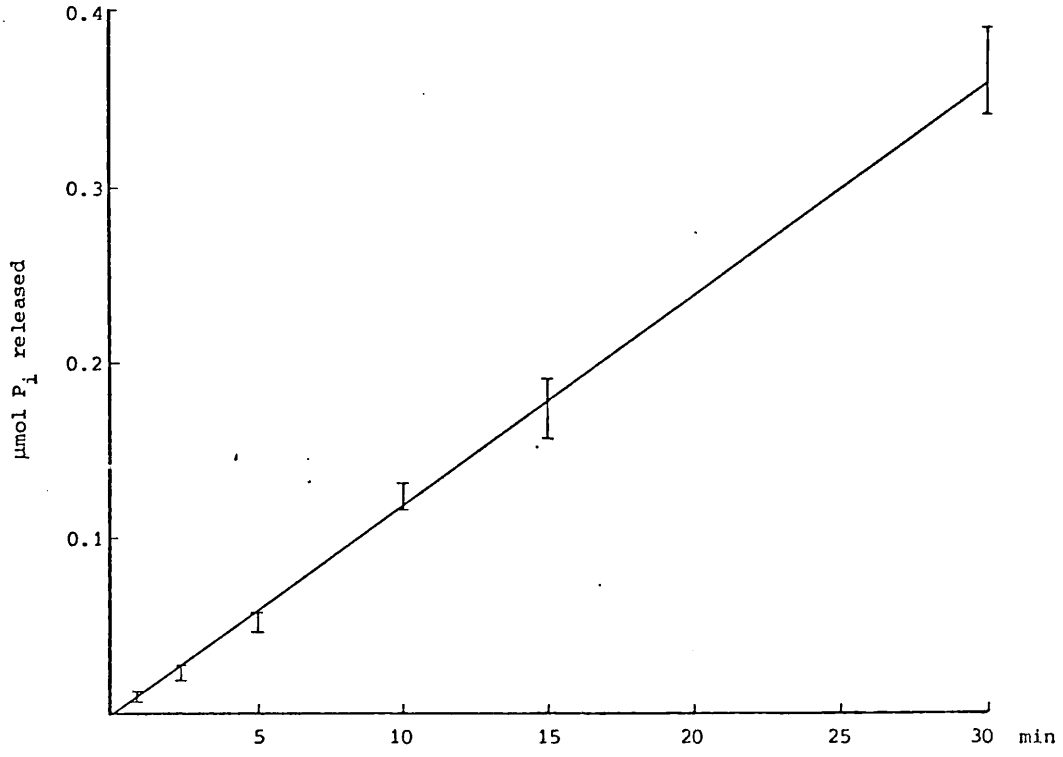


Fig. 4.3.2

4.3 Results

4.3.1 The pH profile of the ATP-dephosphorylating enzyme in the homogenate

The pH profiles of the ATP-dephosphorylating enzyme in the homogenate and of an equivalent amount of ovorubin are shown in Fig. 4.3.1. The pH profile of a reconstituted homogenate is almost indistinguishable from that of ovorubin contained therein.

Fig. 4.3.1 a) The pH profile of the ATP-dephosphorylating enzyme in a 5% (w/v) egg homogenate containing 2.7 mg/ml protein (including 1.4 mg/ml ovorubin)

The experiment was carried out in the presence of 10 mM ATP. Otherwise conditions were as described under 4.2.1.

b) The pH profile of the activity associated with 1.4 mg/ml ovorubin in the presence of 40 mM ATP

For assay conditions see 4.2.1.

Fig. 4.3.2 Release of inorganic phosphate from ATP by a 5% (w/v) egg homogenate pH 6.8, 20 mM ATP, 10 mM $MgCl_2$, 30°C. Final volume 100 µl.

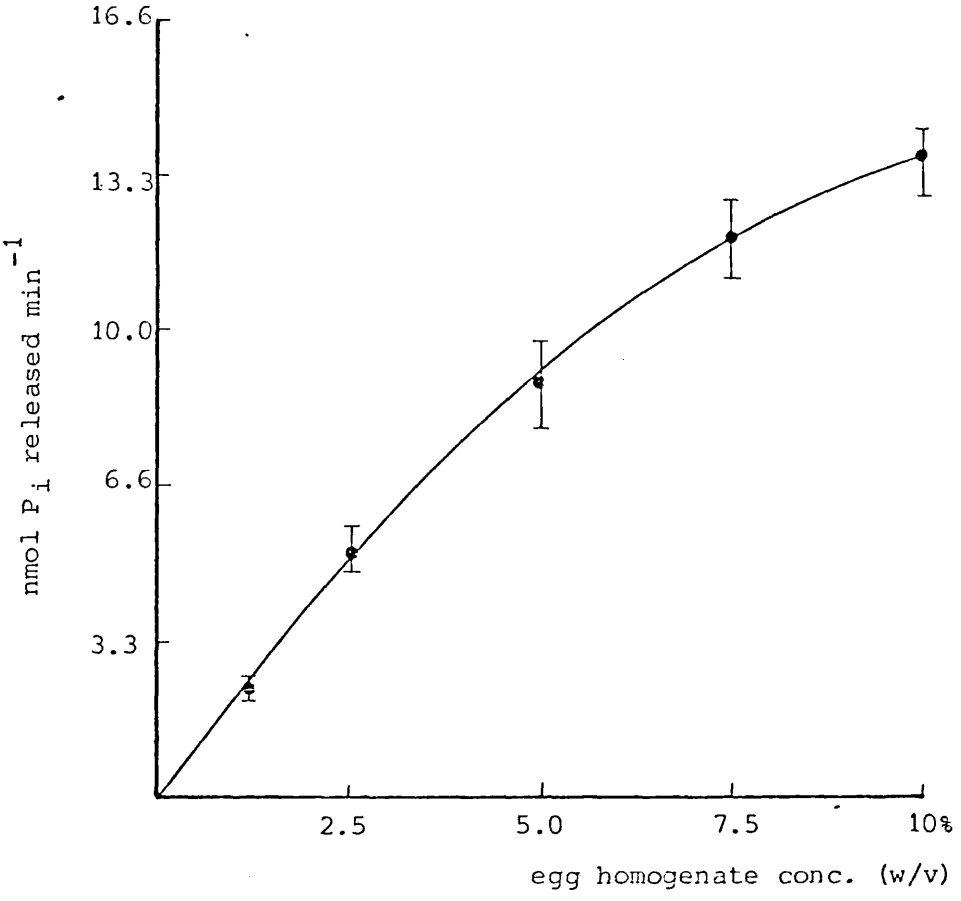

16.6
13.3
10.0
6.6
3.3
nmol P_i released min^{-1}
2.5
5.0
7.5
10%
egg homogenate conc. (w/v)

Fig. 4.3.3

Fig. 4.3.3 The effect of homogenate concentration (w/v) on the release of inorganic phosphate from ATP.

Assay conditions as for Fig. 4.3.2.

4.3.2 The effect of activators and inhibitors on the activity of the ATP-dephosphorylating enzyme

Effectors all at 10 mM conc.	nmol min^{-1} P_i released	Δ%
Control	2.0	
$MgCl_2$	5.8	+190
$CaCl_2$	5.55	+177
KCl	1.66	-17.7
NaCl	1.71	-14.5
KCl + $MgCl_2$	4.9	+145.2
NaCl + $MgCl_2$	5.2	+159.7
KCl + NaCl	1.61	-19.4
NaCl + KCl + $MgCl_2$	4.8	+140.3
10 μM ouabain	1.96	-1.6
NaCl, KCl, $MgCl_2$ + 10 μM ouabain	4.8	+141.9

Table 4.3.1

The effect of some ions on the activity of the ATP-dephosphorylating enzyme

The release of P_i was catalysed by a 5% (w/v) egg homogenate at pH 6.8 in 0.1 M Tris-maleate buffer in the presence of 20 mM ATP at 30°C in a volume of 100 μl. Using a Tris-HCl buffer at the same pH, an almost 90% lower activity was obtained in the presence of $MgCl_2$ as compared to that in Tris-maleate.

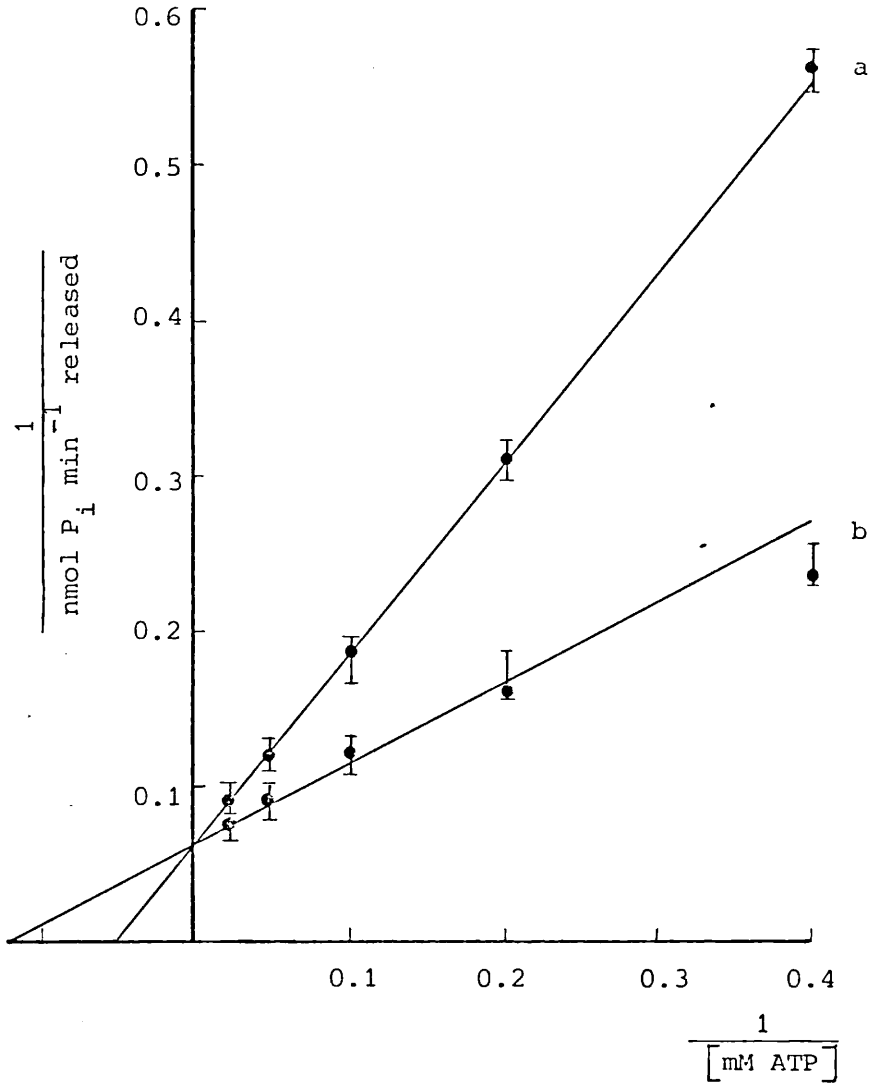


Fig. 4.3.4

Because of the marked activation obtained with $MgCl_2$, the effects of other potential modulators were investigated in the presence of 10 mM $MgCl_2$ under otherwise identical conditions. The results are shown in Table 4.3.2.

Effector	nmol min^{-1} P_i released	Δ%
Control	5.8	-
10 μM oligomycin	9.3	+60.5
100 μM 2:4-DNP	9.35	+61.1
5 mM dithionite	8.8	+51.6
5 mM dithiothreitol	9.1	+56.1
10% methanol	9.25	+59.4
0.1% Na deoxycholate	5.26	-9.4
0.1% Triton X100	4.97	-14.4
Repeated freezing and thawing of the homogenate	5.1	-12.4
Dark	5.74	-1.1

Table 4.3.2

Modulators of the ATP-dephosphorylating enzyme in the presence of 10 mM $MgCl_2$

Assay conditions as shown in Table 4.3.1.

4.3.3 The release of P_i from ATP and ADP catalysed by the homogenate

Fig. 4.3.4 a) Lineweaver-Burk plot for ATP concentrations in the range 2.5-40 mM

b) The same as above, in the presence of 20 mM ADP.

The release of P_i catalysed by a 5% (w/v) egg homogenate was carried out as described in Chapter 4.2.1.

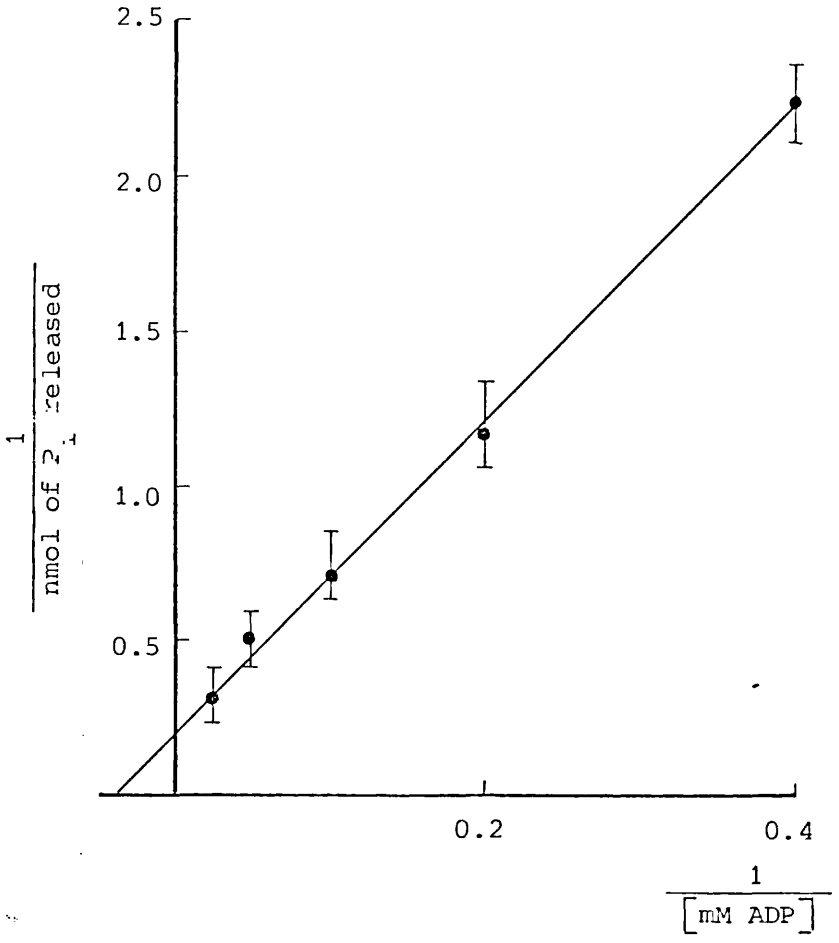


Fig. 4.3.5

The apparent K_m for ATP is in the region of 20 mM. Addition of 20 mM ADP over the concentration range employed for the determination of $K_{m(ATP)}$ leads to an almost fourfold reduction to *ca.* 5 mM, while V_{max} = 17 nmol P_i min^{-1} remains unaltered.

ADP itself has been shown to be a substrate for this enzyme.

Fig. 4.3.5 Lineweaver-Burk plot for the enzymic activity releasing P_i from ADP (concentration range 2.5-40 mM). Otherwise experimental conditions were as for Fig. 4.3.4.

The apparent K_m for ADP is in the region of 28 mM, and V_{max} 6 nmol P_i min^{-1}.

Figs. 4.3.4 and 4.3.5 are based on experiments carried out on four independently prepared egg homogenates and represent 4-6 determinations for every homogenate at each point.

On three other occasions, however, on addition of ADP, there was a decrease in the amount of total inorganic phosphate in the reaction mixture (i.e. as compared to the combined contents of homogenate and reagents).

The amount of P_i in egg homogenates varied between 0.44 and 0.66 µg P_i g^{-1} wet weight in 26 estimations.

	nmol of P_i				
A	0.71	0.68	0.74	0.71	0.68
B	1.06	0.74	0.52	0.42	0.37
C	1.45	1.22	1.13	1.03	1.02
D	-0.32	-0.19	-0.13	-0.1	-0.03
mM ADP in B	40	20	10	5	2.5

Table 4.3.3

Decrease in the level of P_i catalysed by a 5% egg homogenate

A = nmol P_i in 5% (w/v) egg homogenate
B = nmol P_i in reagent blank (ADP, $MgCl_2$, buffer)
C = nmol P_i 1 min. after mixing of A + B
D = Δnmol P_i, i.e. C-(A + B).

The change in P_i content of a 5% (w/v) egg homogenate at pH 6.0,

30°C, 10 mM $MgCl_2$ was measured one minute after the addition of ADP.

The results in Table 4.3.3 represent the means of six determinations obtained from three independently prepared homogenates. These obviously contradict Fig. 4.3.5. However, it is possible that this variation is due to a difference in the stage of egg development; the results vary sporadically between homogenates, although they are consistent within individual preparations.

When both ADP and ATP were used as substrates there was invariably an increase in the amount of inorganic phosphate released as seen from Fig. 4.3.4 and Table 4.3.4.

	nmol P_i				
A	0.74	0.77	0.71	0.74	0.77
B	2.0	1.6	1.39	1.29	1.23
C	14.6	10.6	8.2	8.1	4.26
D	12.6	8.35	5.6	3.2	1.78
C-D	1.94	2.26	2.57	2.58	2.48
nucleotide in reaction mixture			mM		
ADP	20	20	20	20	20
ATP	40	20	10	5	2.5

Table 4.3.4

Release of P_i from ATP and ADP catalysed by a 5% (w/v) homogenate at pH 6.8, 10 mM $MgCl_2$, 30°C one minute after mixing of homogenate and reagents (100 µl final volume).

A = nmol P_i in 5 % (w/v) egg homogenate
B = nmol P_i in reagents (ATP, ADP, $MgCl_2$ and buffer)
C = nmol P_i min^{-1} released by the homogenate in the presence of the reagents
D = nmol Pi min^{-1} released with only ATP as substrate

The results tabled show the mean values of four determinations obtained from the above-mentioned three independently prepared homogenates (Table 4.3.3).

4.3.4 The molecular entity associated with the adenine nucleotide dephosphorylating enzyme

Since the homogenate freed from ovorubin by its adsorption on Con A-Sepharose showed no release of P_i in the presence of ATP, it was decided to investigate the activity of a reconstituted homogenate consisting of 1.4 mg ml^{-1} ovorubin added to the residue of a 5% (w/v) egg homogenate previously freed from the carotenoglycoprotein. In addition the activity of electrophoretically prepared sub-fractions of ovorubin (see 3.2.7) i.e. F_{2c}, F_{oa}, F_{3a} and F_{4a} as well as that of the apoprotein were tested.

	nmol P_i released by							
pH	a	b	c	d	e	f	g	h
5.4	0	0.1	0.1	0	0.1	0	0	0.1
5.6	0	0.3	0.26	0.1	0.3	0	0	0.2
5.8	0	0.2	0.21	0	0.2	0	0	0.15
6.0	0	0.1	0.2	0	0.15	0	0	0
6.2	0	0.2	0.1	0	0.2	0	0.1	0.1
6.4	0	0.5	0.52	0	0.55	0	0.1	0.5
6.6	0.1	0.62	0.65	0	0.73	0	0.1	0.6
6.8	0.1	1.15	1.18	0.1	1.23	0	0.1	1.1
7.0	0.1	1.0	1	0	1.1	0.3	0	1
7.2	0	0.55	0.58	0.1	0.68	0	0	0.56
7.4	0	0.5	0.5	0	0.52	0	0	0.5
7.6	0.1	0.56	0.57	0	0.52	0	0	0.51
7.8	0.1	0.62	0.6	0	0.53	0	0	0.6
8.0	0.2	0.77	0.77	0	0.76	0.1	0	0.7
8.2	0.2	0.81	0.8	0	0.82	0.15	0	0.76
8.4	0.1	0.65	0.6	0	0.62	0	0	0.7
8.6	0	0.36	0.32	0	0.37	0	0	0.32
8.8	0	0.25	0.27	0	0.19	0	0	0.26

Table 4.3.5

The molecular entity associated with the ATP dephosphorylating enzyme

Assay conditions: Buffer systems as described in Chapter 4.2.1. Total reaction volume 100 µl containing 10 mM $MgCl_2$, 40 mM ATP at 30°C. The reaction was stopped after 10 min. and the results tabelled represent the average release of P_i min^{-1} catalysed by:

a) 5% (w/v) egg homogenate freed from ovorubin
b) 1.4 mg ml^{-1} ovorubin
c) a + b
d) 1.4 mg ml^{-1} ovorubin F_{2c}
e) 1.4 mg ml^{-1} ovorubin F_{oa}
f) 1.4 mg ml^{-1} ovorubin F_{3a}
g) 1.4 mg ml^{-1} ovorubin F_{4a}
h) 1.4 mg ml^{-1} apo-ovorubin

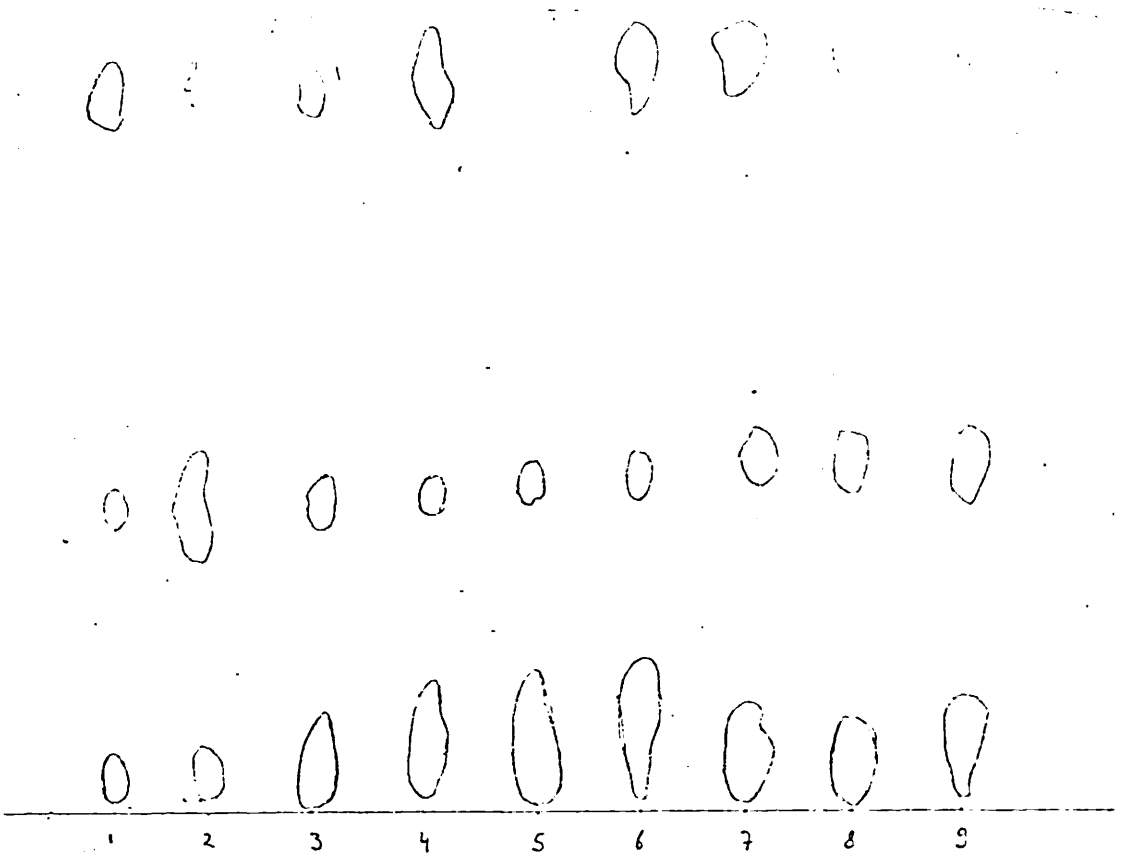


Fig. 4.3.6a

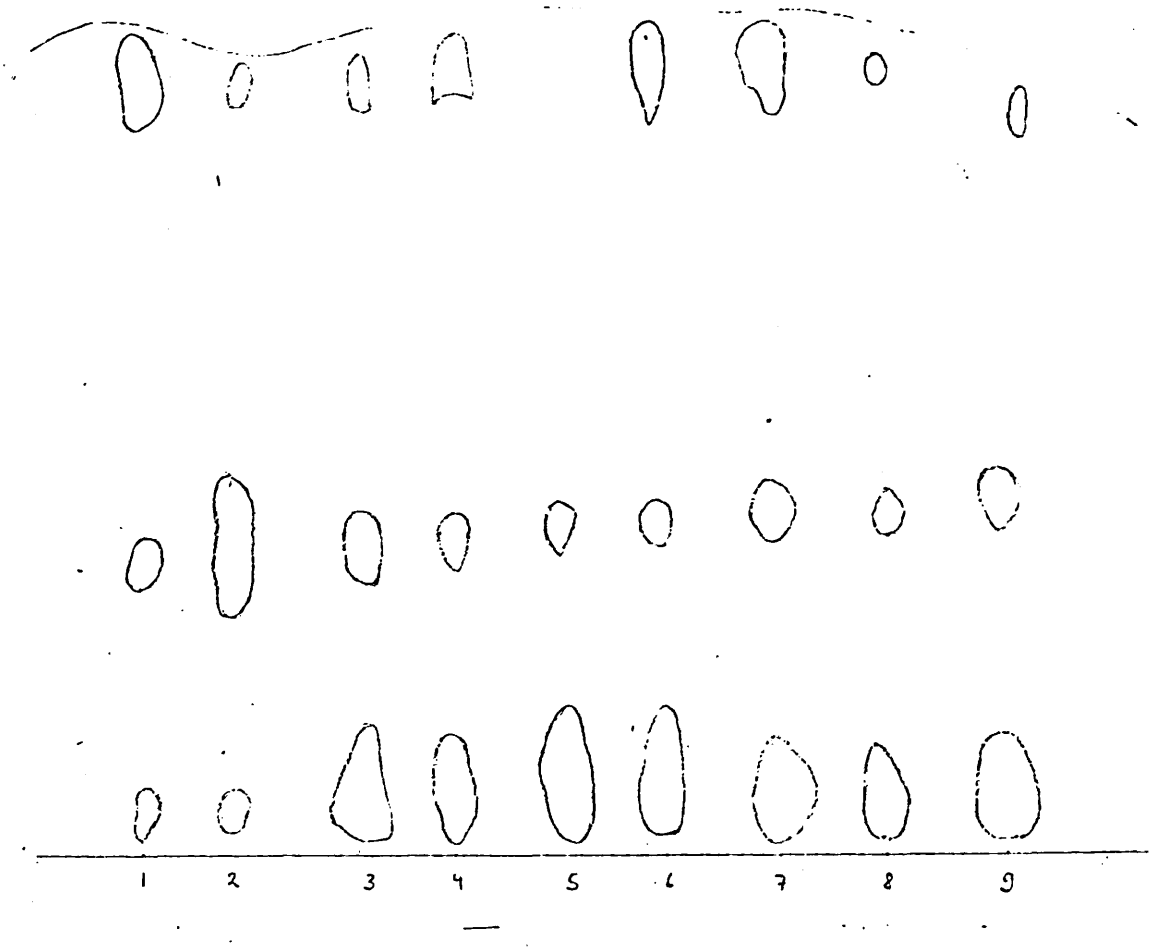


Fig. 4.3.6b

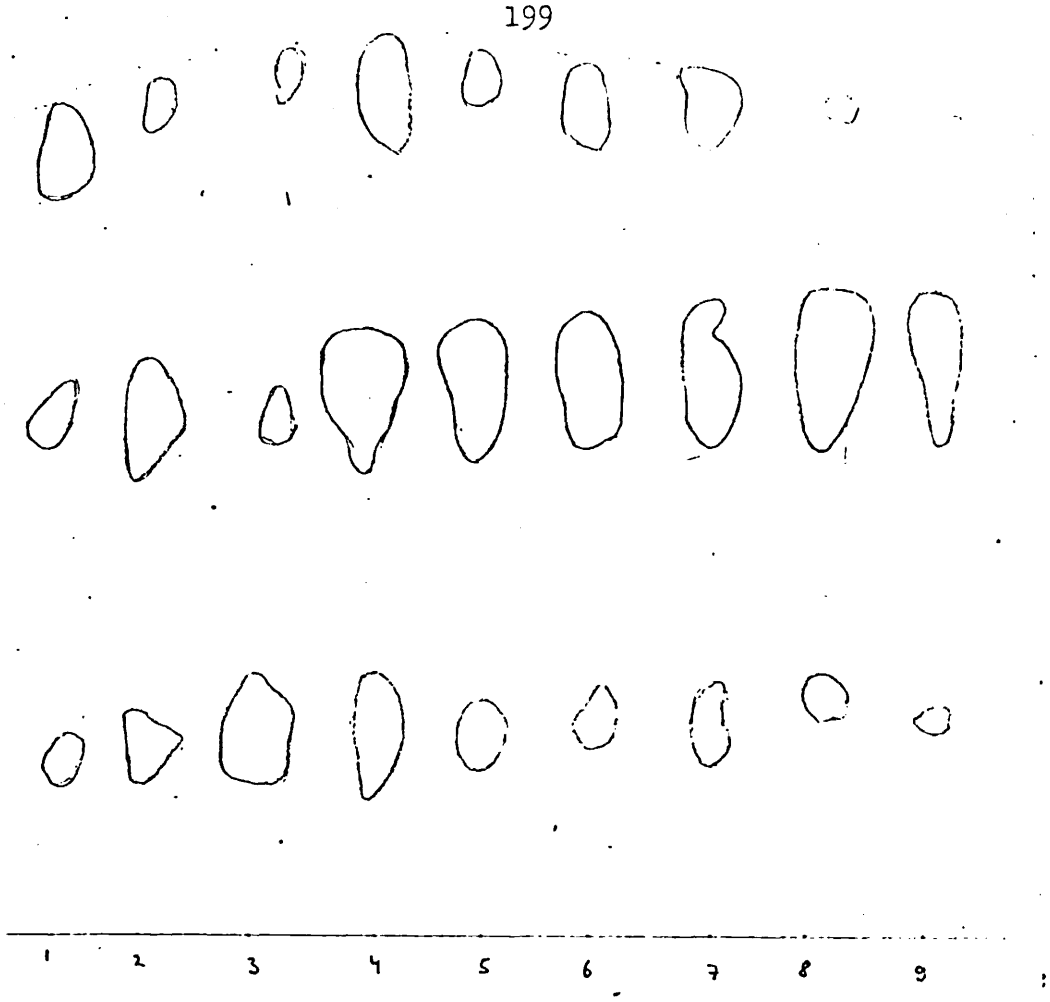


Fig. 4.3.6c

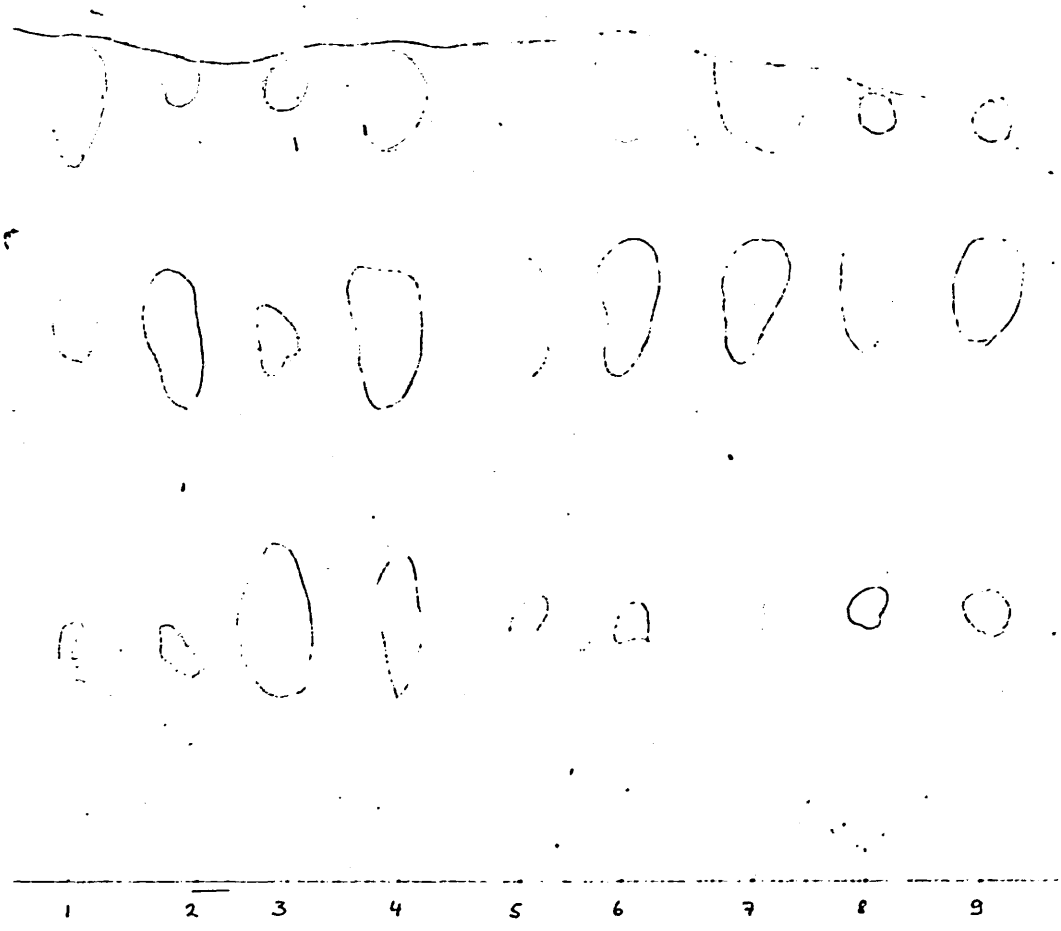


Fig. 4.3.6d

The results in Table 4.3.5 represent the means of four determinations for a-d and h, and the means of three determinations for d-g at pH 5.6, 6.8 and 8.2. All other figures represent a single determination because of the scarcity of material.

4.3.5 Identification of the reaction product

The experiments described above suggest that the phosphatase activity associated with the egg homogenate and with ovorubin is not a true ATPase, since ADP is also an effective substrate for the enzyme.

Fig. 4.3.6a-d show the chromatographic separation of adenine nucleotides by the method of Randerath (1963) as modified by Shirley (1978) using LiCl as a developing agent; the spots were located under a Hanovia lamp. The order of application (left to right) was as follows:

1. AMP standard
2. ADP standard
3. ATP standard
4. 5% egg homogenate
5. the same freed from ovorubin
6. ovorubin
7. ovorubin F_{oa}
8. ovorubin F_{2b}
9. ovorubin F_{4a}

Fig. 4.3.6a Nucleotide analysis obtained after 5 min. incubation of 4-9 in the presence of 40 mM ATP, 10 mM $MgCl_2$ at pH 6.8 and 30°C. The chromatogram was developed with 1.4 M LiCl.

Fig. 4.3.6b Nucleotide analysis after 15 min. incubation with ATP, otherwise conditions as for Fig. 4.3.6a.

Fig. 4.3.bc Nucleotide chromatogram obtained after 5 min. incubation of 4-9 in the presence of 40 mM ADP, 10 mM $MgCl_2$ at pH 6.8 and 30°C. The chromatogram was developed with 2.0 M LiCl.

Fig. 4.3.6d Nucleotide chromatogram obtained after 15 min. incubation of 4-9 with 40 mM ADP. Otherwise conditions were as for Fig. 4.3.6c.

Chromatographic analysis suggests that the final product of the phosphatase activity is AMP, irrespectively of whether homogenate, ovorubin or the ovorubin fraction F_{oa} is used as the source of catalytic power. In Fig. 4.3.6c and d, spot number 4 represents the reaction products of a homogenate in which a decrease in the total amount of P_i was observed; the chromatographic analysis suggests that a synthesis of ATP may occur under conditions not yet clearly defined.

4.4.1 Discussion

As can be seen from Fig. 4.3.1a and b, the pH profile of the phosphatase present in egg homogenates of *P. canaliculata* and that associated with ovorubin shows three distinct pH optima in the region of pH 5,6, 6.8-6.9 and 8.0-8.2. The amounts of P_i mg^{-1} protein hr^{-1} released from the egg homogenate at these pH optima are 0.145, 1.33 and 0.9 µmol whereas the corresponding figures for ovorubin are 0.08, 0.5 and 0.39 µmol, i.e. less than half of those for the homogenate. (Note : substrate concentration employed for homogenate assays was 10 mM ATP as against 40 mM ATP when ovorubin was the source of catalytic activity). Table 4.3.5 shows that the ATPase activity of a reconstituted egg homogenate is virtually indistinguishable from that associated with ovorubin, suggesting that the active factor co-purifies with ovorubin, although a high percentage is lost during the purification procedure. Experience has shown that some 25% of ovorubin purified either by adsorption chromatography on Con A-Sepharose or calcium phosphate gel cannot be removed by the eluting procedure employed, indicating tight binding and the possibility that the active principle has a high affinity for both these adsorbents. Its association with ovorubin may confer the observed high-affinity properties on the complex. Electrophoretically prepared subfractions of ovorubin with the exception of F_{oa} do not catalyse the release of P_i from ATP and analysis by SDS electrophoresis shows that inactive fractions lack a protein component characterised by a positive PAS reaction and an apparent molecular weight in excess of 150,000. This glycoprotein may hence be the active principle. This is supported by the observation that it is part of chromatographically prepared ovorubin and co-purifies quantitatively with the F_{oa} fraction.

The most important inhibitor of ATPases from mitochondria or chloroplasts is ADP. The inhibition is competitive with a K_i in the region of 30 µM. In contrast, the enzyme present in homogenates from *P. canaliculata* eggs is not subject to such inhibition, as can be seen from Fig. 4.3.2 which shows the release of Pi from ATP over a period of 30 min by a 5% (w/v) homogenate at pH 6.8. Fig. 4.3.3 indicates, however, that the rate of hydrolysis does not increase linearly with the homogenate concentration. Since Fig. 4.3.2 renders direct product inhibition or substrate depletion an unlikely explanation, the possibility

should be considered that we are observing a proximity effect due to some factor that regulates the enzymic activity at higher homogenate concentrations. Table 4.3.1 shows that the release of P_i catalysed by the homogenate at pH 6.8 is stimulated 11-12 fold by 10 mM $MgCl_2$ and $CaCl_2$, whereas NaCl and KCl separately and in combination cause between 15 and 20% inhibition in the absence of $MgCl_2$. This inhibition is even more pronounced in the presence of $MgCl_2$, where it ranges between 30 and 50%. The reaction is completely unaffected by the presence or absence of ouabain , thus ruling out the possibility that we are dealing with a transport ATPase of the type found in plasma membranes.

At pH 6.8 and in the presence of 10 mM $MgCl_2$, 5 mM dithionite, 5 mM dithiothreitol, 10% methanol and 10 µM oligomycin enhance the rate of hydrolysis by approximately 50 to 60%. 0.1% Na deoxycholate, 0.1% Triton X100 and repeated freezing and thawing cause between 10 and 15% inhibition, while the presence or absence of diffuse daylight is virtually without effect.

In comparison with the effects of freezing and thawing and the pH-activity profile, the ATP-dephosphorylating enzyme in the egg homogenate of *P. canaliculata* and that associated with ovorubin resemble more closely that of rat brain mitochondria (Lagnado *et al.*, 1959) which is unaffected by freezing and thawing, and displays pH optima at 5.9-6.1, 7.1-7.2 and 8.3-8.5 while the ATPases from liver mitochondria (Myers and Slater, 1957) show a marked degree of latency in that they display activity only after disruptive procedures like freezing and thawing; their optima are found at higher pH, i.e. 6.3, 7.5 and 8.5.

Like the rat liver mitochondrial ATPase and unlike rat brain mitochondrial ATPase, the enzyme in egg homogenates is greatly stimulated by the uncoupling agent 2:4 DNP (ca. 60% at pH 6.8 in the presence of 10 mM $MgCl_2$).

ATPases from rat liver and yeast mitochondria show an increase in activity of up to fivefold in maleate buffers. Similar results were obtained with the enzyme from *P. canaliculata*, although in this case the activation was only in the region of 90%.

Mitochondrial ATPases of all tissues investigated have been resolved into a number of subunits, and many of the properties observed have been traced to the presence or absence of such subunits.

Isolated and membrane-bound ATPases are often affected in different ways by a series of compounds, some of which inhibit the soluble form of the ATPases little, if at all, but do inhibit membrane-bound or particulate forms of the enzyme. At least three examples are known of small proteins which, apparently in close association with the ATPase in an energy-transducing membrane, confer on the enzyme sensitivity to specific inhibitors.

All the soluble forms of the mitochondrial ATPase that have been investigated are insensitive to the antibiotic oligomycin, which inhibits oxidative phosphorylation.

An essential protein component, called the oligomycin sensitivity conferring protein OSCP or FC, has been isolated from beef heart and baker's yeast mitochondria (Bulos and Racker, 1968; Tzagoloff and Meagher, 1971). It appears to participate in binding the enzyme to the membrane. In the cases observed, oligomycin inhibits phosphorylation in such a way that electron transport is also inhibited, a phenomenon that is reversed by 2,4-DNP. It has been concluded that oligomycin acts primarily on the energy-coupling mechanism. At this stage, nothing can be said concerning the presence of an oligomycin-combining protein in the egg homogenate, especially since the nature of the ATP-dephosphorylating enzyme in the homogenate is only inferred from indirect evidence.

Many of the properties of the enzyme suggest that one is dealing with a different type of protein altogether, which nevertheless seems to share some properties with energy-coupling ATPases of mitochondria and chloroplasts.

In contrast to mitochondrial and chloroplast ATPases, which are competitively inhibited by ADP, the enzyme present in homogenates from *P. canaliculata* can utilise this as a substrate.

The activation of energy-transducing ATPases by alcohols appears either to be due to the removal of the inhibiting ADP that is bound to the enzyme, or may possibly stem from an alteration of the ADP-binding site (Penefsky and Warner, 1965).

The observation that ADP is a substrate and not an inhibitor for the enzyme, renders it at first sight difficult to explain the activation by alcohols in analogy to the stimulation of mitochondrial and chloroplast ATPases by these compounds. It should, however, be considered that alcohols may destabilise the enzyme-product complex.

The snail egg phosphatase is stimulated by dithionite. Myers and Slater (1957) find that the ATPase from rat liver mitochondria is activated by this reducing agent, although a mechanism for this has not been proposed by the authors, who find that other reducing agents do not produce the same effect as dithionite. The ATPase from plant chloroplasts is greatly stimulated by dithiothreitol (DTT) (Farron and Racker, 1970), which is thought to modify the coupling factor *in situ*. It is found that the SH groups of DTT are required for the activation of this ATPase, although it is considered unlikely that they play a specific role in the hydrolysis of ATP.

As far as activation by the above-mentioned substances is concerned, there is a close resemblance between energy-coupling ATPases and the enzyme from *P. canaliculata* eggs.

The fact that ADP is not a potent inhibitor but a substrate and the magnitude of the apparent K_m for ATP, which is some 100 times higher than that obtained with most energy-transducing ATPases, strongly suggests a different class of enzyme. Also, as can be seen from Fig. 4.3.6c-d, the major reaction product with both ATP and ADP as substrate seems to be AMP, whether homogenate, ovorubin or ovorubin F_{oa} is used as source of catalytic power. The enzyme therefore somewhat resembles an apyrase found in potato (Lee and Eiler, 1951) and shares some properties with an enzyme that occurs in mitochondria of insect muscle (Sacktor, 1953). Both are Mg^{2+}-stimulated ATPases catalysing

$$ATP \longrightarrow AMP + 2P_i$$

There is no suggestion that the latter enzyme is involved in the energy-transducing machinery. Like the enzyme from *P. canaliculata*

it is activated by Mg^{2+}. However, there is no activity if Mg^{2+} is replaced by Ca^{2+}, Na^{+} or K^{+}. Its optimum pH lies at pH 7.8-8.0. The enzyme shows substrate inhibition, the optimum concentration being 1.6 mM for ATP and 1.4 mM for ADP. ATP is hydrolysed at about ten times the rate of ADP. Inorganic pyrophosphate and other organic phosphate and pyrophosphate esters are not split. In contrast, preliminary investigations on the enzyme from the egg homogenates suggest that it may act as a non-specific phosphatase, since it appears to catalyse the hydrolysis of disodium phenyl-phosphate (O. Kleinova, personal communication). It is, however, somewhat surprising that when ATP is used as substrate very little ADP accumulates, which one would expect if the γ-and β-phosphate groups were removed sequentially, especially in view of the fact that ADP is a poorer substrate and that the ADPase activity of the enzyme may be inhibited by high concentrations of ATP, as suggested by the results in Table 4.3.4. Since there is no evidence for the release of pyrophosphate, the possibility must be considered that on hydrolysis of ATP, the ADP formed binds to the enzyme, thereby being largely removed from the solution. If this were so, it could also explain the earlier mentioned activation of the ATPase by alcohols, which are thought to stimulate energy-transducing ATPases by removing the ADP that is bound to the enzyme.

When ADP is used as substrate, there is sometimes a decrease in P_i in the reaction mixture, amounting to some 71 nmol mg^{-1} protein hr^{-1} at pH 6.8 in the presence of 40 mM ADP. This could be considered as suggestive evidence that Pi has been incorporated into some other molecule. In view of the synthesis of ATP observed in some homogenates (see chapter 2), the chances are that part or all of the P_i that has been removed from solution is incorporated into ATP. This possibility is further supported by spot number four in Fig. 4.3.6c and d which indicates a certain increase in the amount of ATP after 5 and 15 min incubation of the homogenate with 40 mM ADP at 30°C.

Although solutions of AMP, ADP and ATP each contained traces of the others, there appears to be a definite increase in the amount of ATP found in reactions catalysed by some homogenates in the presence of ADP with respect to the control spot of this substrate (2). This is not observed in reactions where the source of catalytic activity is ovorubin or its catalytically active sub-fraction, or homogenates in which no decrease in the level of P_i is observed.

Although chromatographic analysis of these homogenates appears to suggest the presence of an adenylate kinase catalysing

$$2\ ADP \longrightarrow AMP + ATP$$

it is unlikely that such a reaction can account for the observed appearance of AMP and ATP, which in the homogenate is accompanied by a change in the concentration of P_i.

No analysis was made of the reaction product of a reconstituted homogenate; it is therefore impossible to say whether the apparent synthesis of ATP is dependent on the reversal of an ATPase reaction associated with ovorubin and whether, in fact, we are dealing with a situation analogous to that observed in partially uncoupled mitochondria. If this were the case, it might explain the apparent stimulation by oligomycin of the release of P_i from the homogenate, since this substance inhibits phosphorylation and therefore its action on the homogenate could explain the observed increase in P_i in its presence.

There is a considerable likelihood that, since experiments have been performed on eggs at somewhat different stages of development, the synthesis of ATP is due to the appearance of mitochondria from the embryo. Unfortunately, the number of mitochondria in the embryonic cells is not known. In view of

a) the considerable variation in the number of mitochondria obtained from different sources (Lehninger, 1965, mentions a variation from 10 to 200,000 per cell depending on species and tissues), and

b) the ignorance of the states (Chance and Williams, 1955) of any mitochondria present or the possible degree of uncoupling,

it is impossible to make a realistic assessment of a contribution from embryonic mitochondria. In addition, it appears very doubtful that mitochondria would be able to withstand the ionic conditions of the experiment in an active state, although spontaneous formation of phosphorylating phospholipid vesicles cannot be excluded.

It appears more likely that the apparent synthesis of ATP may be quite unrelated to energy-coupling ATPases, but nevertheless dependent in some other way on the presence of ovorubin. Cheesman (unpublished observations) has obtained tentative evidence for the presence of a glycolytic system in egg homogenates.

CHAPTER 5

OVORUBIN AND ITS INTERACTION WITH HELICORUBIN P AND CYTOCHROME C

5.1. Introduction

In Chapter 2 evidence has been presented that removal of ovorubin from the homogenate leads to a rapid oxidation of the cytochrome. This is less pronounced when the homogenate is centrifuged at 10,000 g than at 2000 g, suggesting that a particulate helicorubin P oxidase is present and, furthermore, that ovorubin is in some way involved in the maintenance of the redox state of helicorubin P. In eggs collected within 1-14 hrs after laying and stored for up to 18 months at -20°C in a deep-freeze compartment, the helicorubin is between 70 and 95% in the reduced form, with higher values in freshly laid, unstored eggs. As shown in Chapter 2, the homogenate contains an enzyme catalysing the transfer of reducing equivalents from NADH, NADPH, GSH and ascorbate to helicorubin P. On removal of ovorubin from the homogenate, this activity was diminished to varying extents, indicating that the active principle is associated with ovorubin. Further experiments showed that when ovorubin was returned to these homogenates, activity was completely restored.

After purification of ovorubin and helicorubin P was achieved, experiments with ovorubin, its apo-protein and electrophoretically purified fractions of ovorubin designated as F_{4a} and F_{2c} (the latter two had been kept in the deep-freeze for 18 months) showed that they could catalyse the reduction of purified helicorubin P in the presence of the reducing substances listed above. In these reactions cytochrome *c* from *Candida krusei* or horse heart can replace helicorubin P.

Appropriate controls indicated that purified helicorubin P or cytochrome *c* from the above-mentioned sources is also slowly reduced by ovorubin and its apoprotein in the absence of added reducing equivalents. Further experiments have shown that when ovorubin is added at pH 7.0 to the oxidised cytochrome, the latter is partially reduced in the dark; in visible light, the reduction is virtually complete. With the carotenoid-free apoprotein, partial reduction occurs either in the light or in the dark, to a degree similar to that found with the carotenoprotein in the dark.

These observations suggested that two centres capable of transferring reducing equivalents to cytochrome *c* are part of the ovorubin molecule. It seems, moreover, that one of these centres is influenced by visible light, and is in some way associated with or dependent on the carotenoid moiety.

However, in view of the finding that a cytochrome of the *b* or *c* type is the electron acceptor, the possibility that the production of a superoxide anion by ovorubin is responsible for the observed effect, had to be taken into consideration.

One-electron reduction of elementary oxygen (O_2) produces the superoxide anion, $O_2^{\cdot-}$ which shows an absorption maximum in the 240-260 nm region (Bielski and Gebicki, 1970).

The rate constant for the reaction between ferricytochrome *c* and superoxide at pH 7.8 is about 6×10^5 M^{-1} sec^{-1}. (McCord *et al.*, 1977). In view of the fact that electron transport from NADH and NADPH to cytochrome *c* is partially inhibited by amytal and rotenone, there was a considerable chance that a group of the flavin type was involved in the reaction sequence. Absorption spectra of ovorubin and its apoprotein provide no evidence, however, for the existence of a flavin group of the classical type. It is known that free dihydroriboflavin reacts non-enzymically in seconds and reduced flavin dehydrogenase reacts even faster with molecular oxygen to form the superoxide anion $O_2^{\cdot-}$.

Experiments by Patel and Willson (1973) have compared the rate of reaction between $O_2^{\cdot-}$ and quinone derivatives as well as the reaction between the radical anion and oxygen. They show that when the redox potential of a quinone-hydroquinone couple drops below zero volts, the rate of reaction between the quinone and $O_2^{\cdot-}$ becomes very small. Their data suggest that only substances with strongly reducing "semiquinone" intermediates are able to form $O_2^{\cdot-}$ by reaction with oxygen.

Superoxide can act as a one-electron reductant towards a variety of substances. As already mentioned, cytochrome *c* is commonly used as a detector of $O_2^{\cdot-}$. Photochemical generation of $O_2^{\cdot-}$ by the

spontaneous re-oxidation of photoreduced flavin, coupled to the reduction of cytochrome *c*, was first observed by Massey *et al.* (1969). The reaction is inhibited by various metal ion-containing superoxide dismutases (SOD) that catalyse the following reaction :

$2\ O_2^{\cdot-} + 2H^+ \longrightarrow O_2 + H_2O_2$ (McCord and Fridovich, 1968).

The inhibition of the reduction of cytochrome *c* by SOD is therefore considered as evidence for the formation of the radical.

5.2 Methods and Materials

Ascorbic acid, cytochrome *c* from horse heart, reduced glutathione, KCN, sodium azide, NADH and NADPH were all obtained from BDH Chemicals Ltd. Cytochrome *c* from *Candida krusei* and horse heart, xanthine oxidase, superoxide dismutase (SOD) hypoxanthine, heptylquinolineoxide, antimycin and rotenone were purchased from Sigma Chemical Company, while Na amytal was obtained from Eli Lilly and Co.

5.2.1 Reduction of ferricytochrome *c* by ovorubin in the presence of oxidisable substrates.

The concentration of cytochrome *c* in all assays was determined from the absorbance of its dithionite-reduced form at 550 nm. The increase in reduction of cytochrome *c* by ovorubin, its apoprotein, or electrophoretically purified subfraction F_{2a} or F_{4a} in the presence and absence of NADH, NADPH, GSH and ascorbate was obtained from measurements of the difference spectrum at 550 nm ($\Delta\varepsilon_{550}$ = 20 x 10^3 $M^{-1}cm^{-1}$), or at 418 nm ($\Delta\varepsilon_{418}$ = 70 x 10^3 $M^{-1}cm^{-1}$ (McCord *et al.*, 1977) between the dithionite-reduced cytochrome in the reference cell and the cytochrome in the reduction assay in the test cell.

All reactants were made up in 0.05M potassium phosphate buffer pH 7.0. The assay was performed in 1 ml 0.05M potassium phosphate buffer at pH 7.0 in a 1 cm cuvette thermostated at 30°C. The reaction mixture contained 3 x 10^{-6}M ovorubin, apoprotein or an electrophoretically purified subfraction. 0.1 ml aliquots of substrate were added in order to obtain a final concentration varying from 0.062 to 2 mM in the case of NADH, NADPH and GSH

and 0.015-2 mM for ascorbate. The mixture was equilibrated for 10 min at 30°C and the reaction was started on addition of 0.1 ml of 3 x 10^{-4}M ferricytochrome *c* (98% in the oxidised state). The progress of the reaction was monitored for 10 min by measuring the change in difference spectrum in relation to the reference cell, which contained equal concentrations of all reactants apart from the substrate, which had been replaced by an equivalent amount of 1 mM Na dithionite.

Two sets of control experiments were carried out at the same time :

a) The effect of the above-mentioned oxidisable substrates was measured in the same fashion in the absence of ovorubin, which was replaced by 0.1 ml buffer.

b) The effect of ovorubin was determined in the absence of substrates. The rate of each control reaction was deducted from the overall rate and the results shown in Fig. 5.3.1 represent the total rate - (a + b).

The effects of 2 mM Na amytal, 30 µM rotenone, 1 µM antimycin, 2 µM heptylquinoline oxide, 1 mM Na azide and KCN (all final concentrations) were measured by replacing 0.1 ml of buffer by an equivalent volume of solution containing the inhibitor in the reference and test cells, under otherwise unchanged assay conditions. Antimycin and rotenone were dissolved in 50% (v/v) ethanolic buffer at concentrations of 10 µM and 300 µM respectively. The final concentration of each reducing agent was 0.2 mM.

5.2.2 Reduction of helicorubin P by ovorubin in the absence of substrate.

Controls in the absence of oxidisable substrates showed that ovorubin can reduce helicorbin P or cytochrome *c*. Preliminary experiments to confirm this were carried out at 25°C in 0.1M Tris-HCl buffer pH 7.0, and dual compartment cells (Helma No. 237, path length 0.4375 cm) were used to follow the progress of the reaction, which was started by mixing ovorubin and helicorubin P in one compartment of the cell, while the other

contained buffer; in cases where difference spectra were recorded against artificially reduced or oxidised samples, this latter was replaced by buffer containing either 1 mM Na dithionite or Na persulphate. The final concentration of ovorubin was 0.8 mg ml^{-1} (2.4 x 10^{-6} M) and that of helicorubin 0.087 mg ml^{-1} (5.4 x 10^{-6} M). At the start of these experiments helicorubin P was 90% oxidised. Controls were carried out, in which ovorubin and helicorubin P were placed in different compartments of the cell. The progress of the reaction was followed spectrophotometrically by recording the difference spectra of both mixed and unmixed samples against the corresponding dithionite-reduced and persulphate-oxidised controls. The experiments were carried out in diffuse light.

5.2.3 The effect of light on the reduction of cytochrome *c* by ovorubin and its apoprotein.

These preliminary observations were followed by investigating the effects produced by ovorubin and its apoprotein, both freshly prepared by the method of Cheesman, on the reduction of cytochrome *c* from *Candida krusei* in the light at 5 W m^{-2}, and in the dark in blackened tubes which were covered with aluminium foil. All reactants were made up in 0.1 M Tris-HCl buffer pH 7.0, and the experiment was carried out at 30°C. The final concentrations of ovorubin and its apoprotein were 1.3 mg ml^{-1} (4 x 10^{-6}M), cytochrome *c* 0.15 mg ml^{-1} (1.2 x 10^{-5}M), those of Na amytal and rotenone 2 mM and 30 mM respectively. The reaction assay contained 0.8 ml of buffer, 0.1 ml of ovorubin or its apoprotein and the reaction was started by addition of 0.1 ml cytochrome *c*. The initial reduction of cytochrome *c* (26% red) immediately after mixing of the reactants was determined by measuring the difference spectrum of the above against fully reduced and oxidised reference controls, which were prepared by replacing 0.1 ml of buffer by the equivalent quantity of 1 mM Na dithionite or Na persulphate. The progress of the reaction was followed by measuring the change in difference spectrum of the test cell against the fully reduced control. The effect of inhibitors was studied by replacing 0.1 ml

of buffer by amytal or rotenone in both test and control cuvette.

Dark-incubated assay mixtures were removed from the tubes by means of a blackened syringe and transferred to the cuvette in the spectrophotometer immediately before recording.

In a separate experiment carried out under identical conditions, the interaction of cytochrome *c* with a sample of ovorubin that had been stored at 20°C for 6 months was investigated.

5.2.4 The effect of superoxide dismutase on the reduction of cytochrome *c* by ovorubin in light and dark.

The activity of superoxide dismutase (SOD) was investigated by the method of McCord and Fridovich (1969). The concentration of SOD required to inhibit the rate of reduction of cytochrome *c* by 50% is in the region of 1.2×10^{-7}M. This concentration of SOD was subsequently used in cell assays where the effect of ovorubin on the reduction of cytochrome *c* was measured in the absence of oxidisable substrates.

The experiments were carried out in 1 ml of 0.05M K phosphate buffer at 7.0 in a 1 cm thermostated cuvette at 30°C. The reaction mixture contained 3×10^{-6} M ovorubin or apo-ovorubin and 9×10^{-6}M cytochrome *c* from horse heart. The reduction of cytochrome *c* by ovorubin or its apoprotein in the presence of absence of 1.2×10^{-7} M SOD was followed in the usual way by measuring the change in difference spectrum in relation to the dithionite-reduced sample in the reference cell.

Analogous experiments under anaerobic conditions were carried out in Thunberg cuvettes (Helma No. 193) containing the same concentration of reactants in a volume of 3 ml.

Where appropriate, the reaction mixtures were illuminated at 5 W m^{-2} in an incubator thermostated at 30°C. In these experiments, the cytochrome *c* (Sigma, from horse heart) was 26% reduced.

5.2.5 The effect of reactant concentration on the reduction of cytochrome *c*.

In these experiments cytochrome *c* from horse heart (BDH) was used.

The preparation was initially 98% oxidised. The increase in reduction was obtained from difference spectrophotometry measurements carried out one hour after mixing 0.5 to 5 x 10^{-6} M ovorubin with 3 x 10^{-6} M cytochrome *c* in a reaction mixture buffered at pH 7.0 in 0.05 M K phosphate buffer at 30°C, or after 24 hrs when the reduction of 0.75 to 30 x 10^{-6} M cytochrome *c* in the presence of 3 x 10^{-6} M ovorubin was measured under the same conditions. Illumination and the inhibition by SOD were carried out as described above.

All preparations of ovorubin used in experiments so far described were between 5 and 8 days old.

5.2.6 The effect of ageing of ovorubin on its interaction with cytochrome *c* in the light and dark in the presence and absence of SOD.

As already mentioned, ovorubin which had been kept for several weeks in a deep-freeze compartment had lost its ability to reduce cytochrome *c* in the absence of oxidisable substrates.

There appears to be a gradual change in the capacity and mode of inhibition by SOD of the reduction of cytochrome *c* related to the ageing of ovorubin. Although this needs further systematic investigation, experiments were carried out using a preparation of ovorubin which had been kept for 20 days in a deep-freeze compartment. The effects of light (5 W m^{-2}) and dark on the reduction of cytochrome *c* by ovorubin in the presence and absence of SOD were determined as described in 5.2.4 and 5.

5.2.7 Reisolation of ovorubin after its previous exposure to cytochrome *c* in the light and in the dark.

In order to investigate the reductive capacity of ovorubin after previous exposure to cytochrome *c*, 25 ml of 0.05 M K phosphate buffer pH 7.0 containing 5 x 10^{-6}M ovorubin and 18 x 10^{-6} M cytochrome *c* was exposed to light or kept in the dark for 24 hrs at 30°C. An attempt was made to separate ovorubin from cytochrome *c* by adsorption onto Con A-Sepharose in the presence of M NaCl as outlined in 3.2. A control preparation of ovorubin was treated in the same way without

prior exposure to cytochrome *c*. Elution of ovorubin from the affinity column with 0.3 M α-methylglucose was followed by passage through a Sephadex G10 column for the removal of ions. Spectra of the protein in the reduced and oxidised states were recorded in the usual way.

Use was made for comparative purposes in these experiments of a pinkish brown carotenoglycoprotein from the eggs of *Pomacea insularum*, a species closely related to *P. canaliculata* (T. Pain, personal communication). This protein was isolated on Con A-Sepharose in a manner identical with that employed for ovorubin.

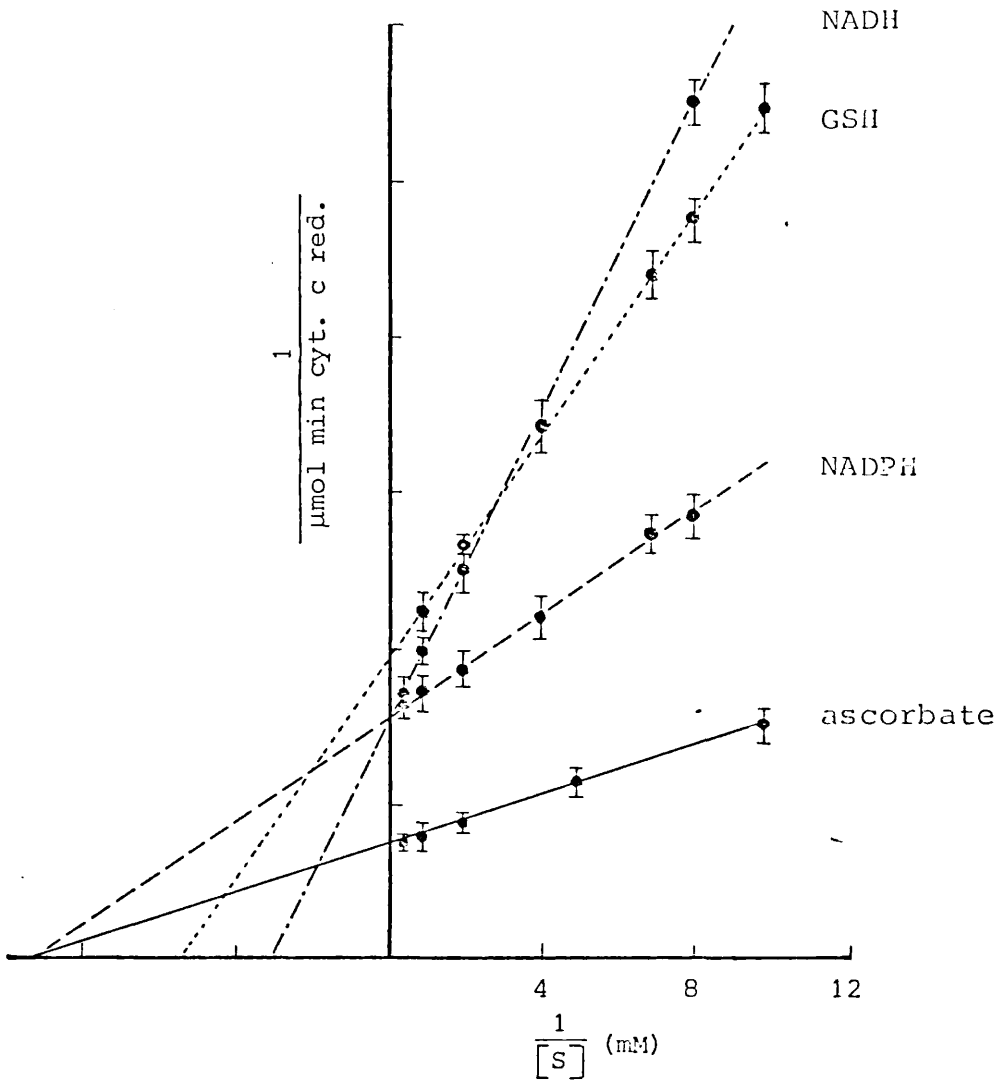


Fig. 5.3.1

5.3 Results

5.3.1. Reduction of ferricytochrome *c* by ovorubin in the presence of oxidisable substrates

Fig. 5.3.1 Lineweaver-Burk plot of the reduction of cytochrome *c* (3×10^{-5} M) by ovorubin (3×10^{-6} M) in 1 ml K phosphate buffer pH 7.0 at 30°C in the presence of:

	Km	V_{max}
ascorbic acid	0.1 µM	0.71 µM min^{-1}
GSH	0.2	0.27
NADH	0.39	0.32
NADPH	0.11	0.32

The results shown are corrected for the non-enzymic reduction of cytochrome *c* by the above listed substrates and by ovorubin in the absence of these substances. They represent the readings obtained from four determinations.

Reduction with the apoprotein and electrophoretically prepared subfraction of ovorubin (F_{4a} and F_{2c}) were carried out in duplicate in the presence of 0.1 mM substrate. The results are essentially identical with those obtained for ovorubin, although the sub-fractions do not reduce cytochrome *c* in the absence of oxidisable substrates.

	Substrate (0.2 mM)			
	NADH	NADPH	GSH	ascorbate
Inhibitor	% inhibition			
2 mM amytal	25	15	1	2
30 µM rotenone	31	23	3	1
0.1 mM antimycin	0	0	0	0
2 µM heptylquinoline oxide	-2	0	0	-3
1 mM Na azide	-5	-3	-2	-2
1 mM KCN	-35	-41	-3	-4

Table 5.3.1

The effects of some electron-transport inhibitors on the rate of reduction of cytochrome *c* by ovorubin in the presence of oxidisable substrates

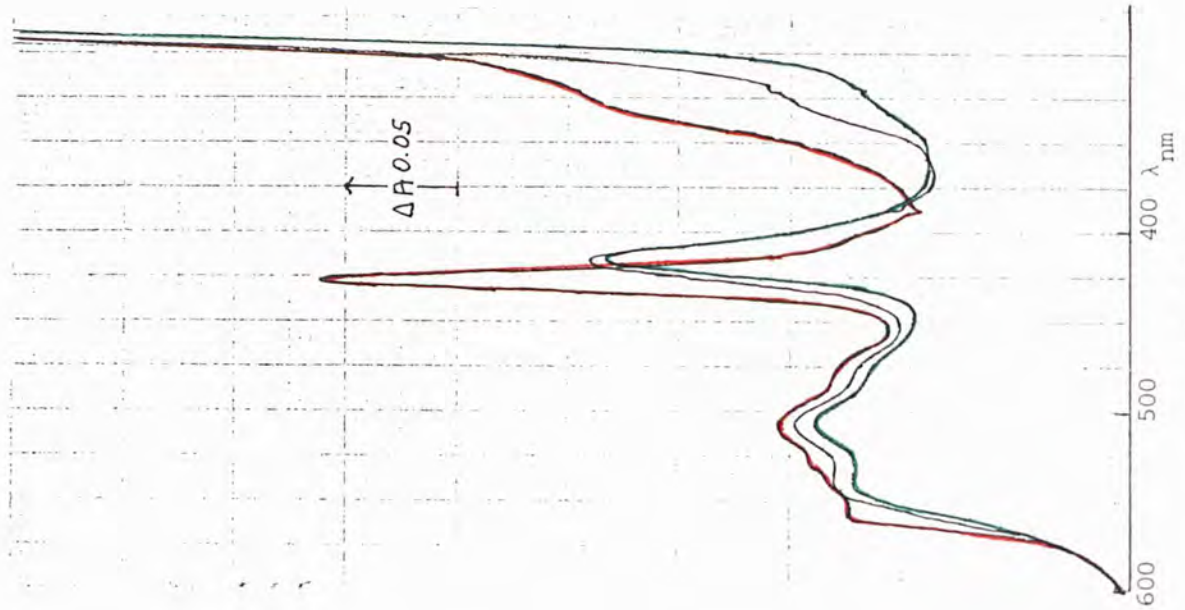


Fig. 5.3.2a

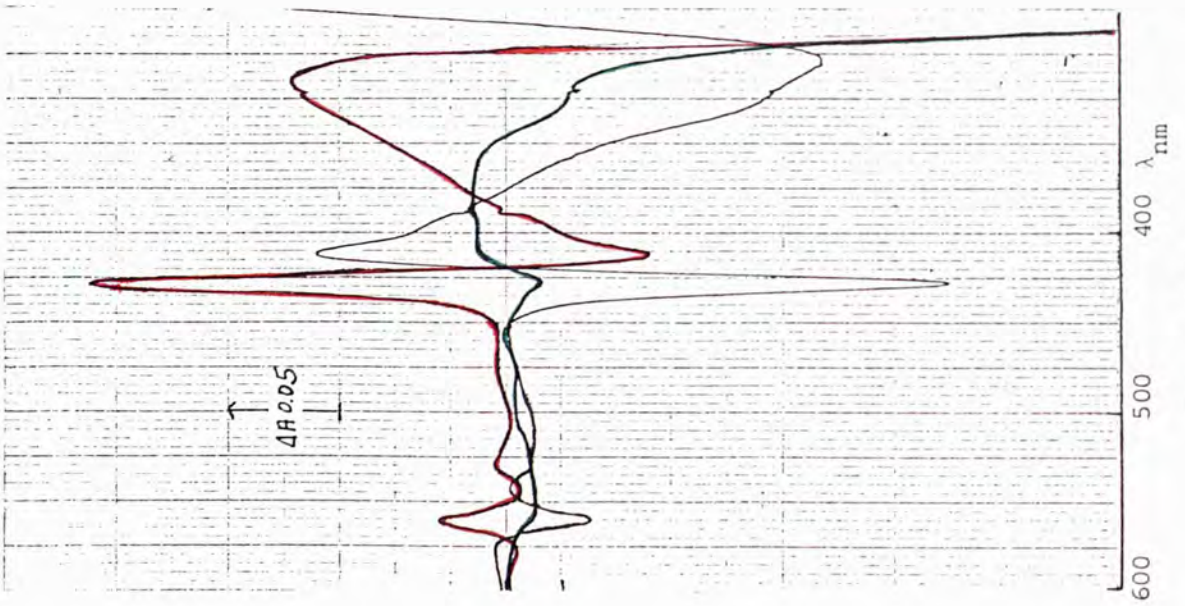


Fig. 5.3.2b

The experiments were carried out as described in Chapter 5.2.1. The effect of the above-listed inhibitors was measured five minutes after the start of the reaction.

5.3.2 Interaction of ovorubin and helicorubin P in the absence of added reductants.

Fig. 5.3.2a Spectrum of 0.8 mg ml^{-1} ovorubin and 0.087 mg ml^{-1} helicorubin P in 0.1 M Tris-HCl buffer, pH 7.0, in dual-compartment cells, path length 0.4375 cm. The two proteins were kept in separate compartments and their spectra were superimposed.

- reduced with 1 mM dithiothreitol.
- oxidised with 1 mM Na persulphate.
- untreated proteins.

Fig. 5.3.2b Difference spectra of the above.

- reduced v. untreated sample
- oxidised v. untreated sample
- oxidised v. reduced sample

The spectra shown were recorded within 10 min of mixing and repeated after 45 min and 26 hrs. No change in the redox state of helicorubin P was observed during this time, i.e. it remains 90% in the oxidised state.

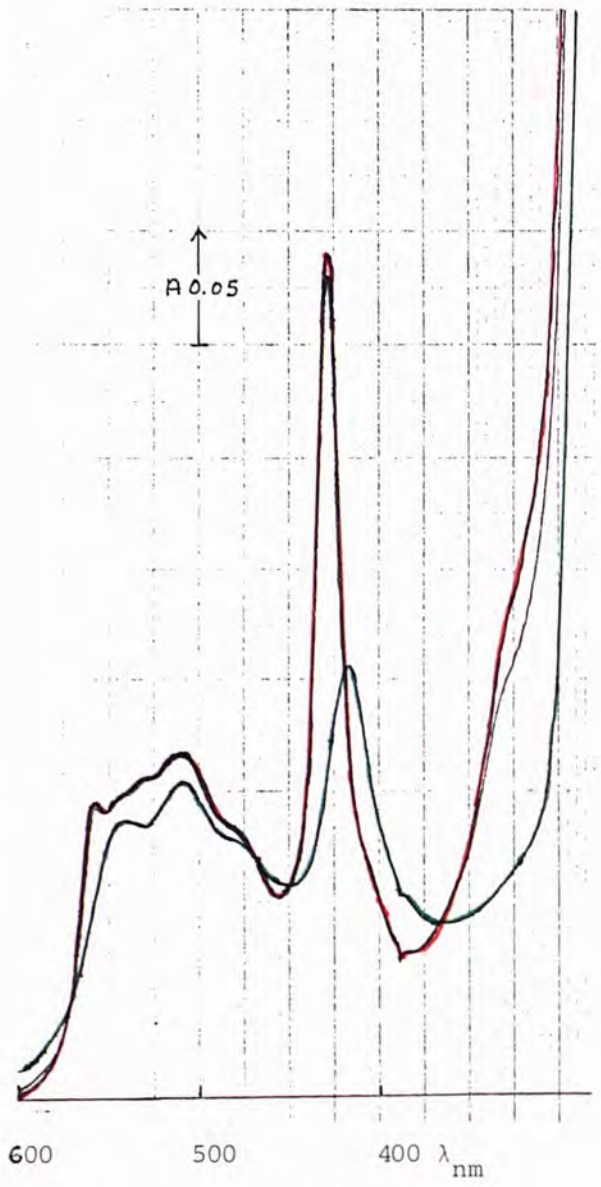


Fig. 5.3.3c

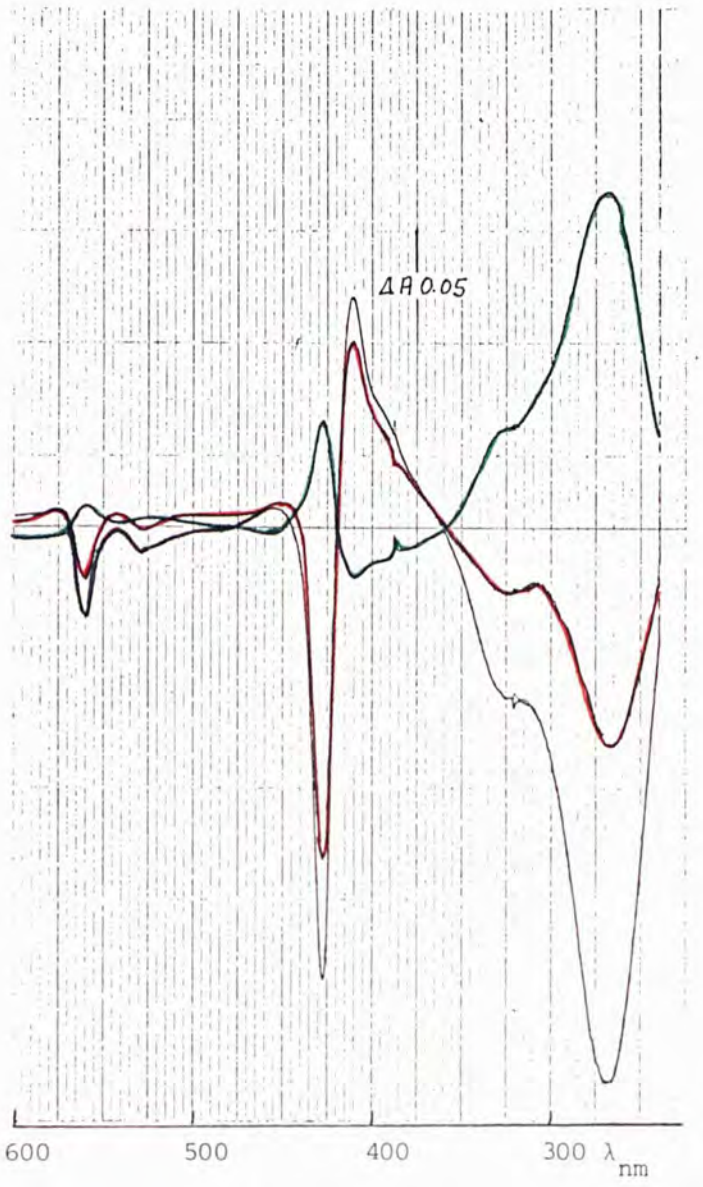


Fig. 5.3.3b

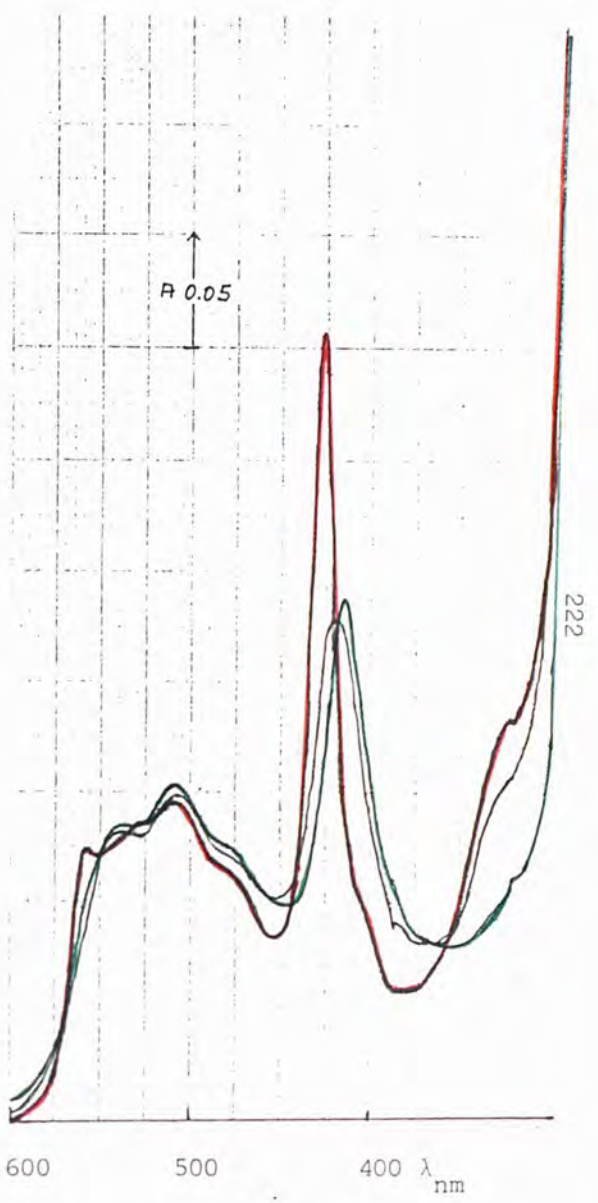


Fig. 5.3.3a

Fig. 5.3.3a Spectrum of 0.8 mg ml^{-1} ovorubin and 0.087 mg ml^{-1} helicorubin in 0.1 M Tris-HCl buffer pH 7.0 in dual-compartment cells, path length 0.4375 cm. The two proteins were mixed in one compartment while the other compartment was filled with buffer containing oxidising or reducing agent where these were appropriate.

- reduced with 1 mM dithiothreitol
- oxidised with 1 mM Na persulphate
- untreated proteins

The spectra shown were recorded 10 min after mixing of ovorubin and helicorubin P. At the start of the reaction helicorubin P was 90% in the oxidised state.

Fig. 5.3.3b Difference spectra of the above recorded 45 min after mixing ovorubin and helicorubin.

- untreated v. reduced sample
- untreated v. oxidised sample
- oxidised v. reduced sample

Fig. 5.3.3c Spectrum recorded 26 hrs after mixing of ovorubin and helicorubin P. Experimental conditions otherwise identical with those in Fig. 5.3.3a and b.

- reduced with 1 mM dithionite
- oxidised with 1 mM Na persulphate
- untreated proteins.

As can be calculated from Fig. 5.3.2b, helicorubin is largely (90%) in the oxidised state at the start of the reaction. On mixing, the degree of reduction increases from 10 to 25% within 45 min and reaches 96% after 26 hrs.

In these experiments, the molar ratio of ovorubin : helicorubin P is in the region of 2.25. Within 45 min and 26 hrs 0.34 and 1.95 moles of reducing equivalents are transferred from ovorubin to helicorubin P.

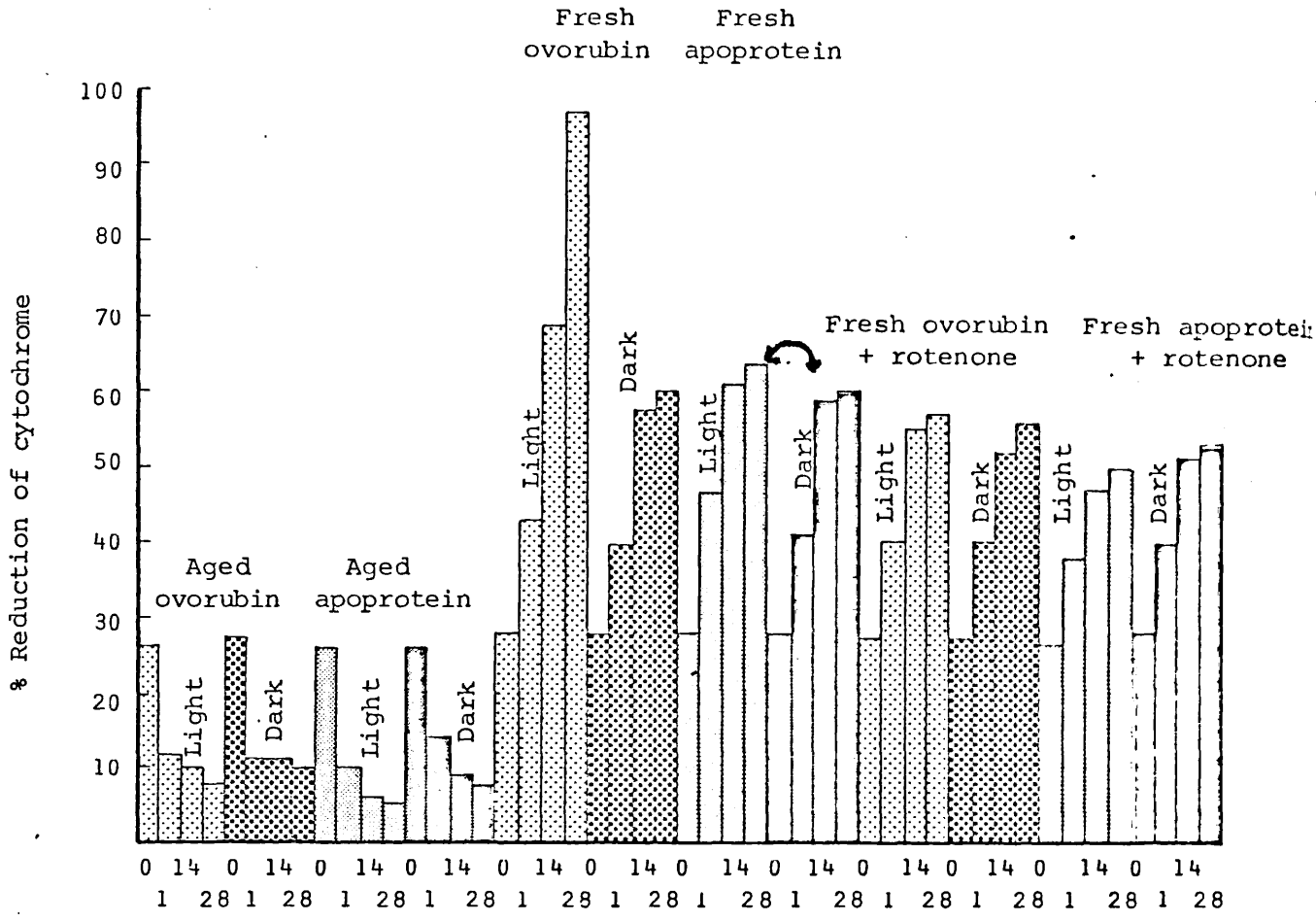

Fresh ovorubin
Fresh apoprotein
Fresh ovorubin + rotenone
Fresh apoprotein + rotenone
Aged ovorubin
Aged apoprotein
Light
Dark
% Reduction of cytochrome
100
90
80
70
60
50
40
30
20
10
0 14 0 14 0 14 0 14 0 14 0 14 0 14 0 14 0 14 0 14 0 14 0 14 0 14 0 14 0 14
1 28 1 28 1 28 1 28 1 28 1 28 1 28 1 28 1 28 1 28 1 28 1 28 1 28 1 28 1 28
Time (hrs) after addition of ovorubin or its apoprotein to cytochrome c from candida krusei

Fig. 5.3.4

5.3.3 The effect of light on the reduction of cytochrome *c* by ovorubin and its apoprotein

Fig. 5.3.4 Percentage increase in reduction of 1.2×10^{-5} cytochrome *c* from *Candida krusei* observed 1, 14 and 28 hrs after addition of 4×10^{-6} M ovorubin or apoprotein (freshly prepared or aged) in the presence of light (5 Wm^{-2}) or 30 μM rotenone at 30°C in 0.1 M Tris-HCl buffer pH 7.0. For experimental details see 5.2.3.

	Initial degree of reduction of cytochrome *c*	Inhibitor	Mol of reducing equivalents transferred to cyt. *c* after hrs.			
			1	14	28	
Cyt. *c* + Ovorubin		-	0.51	1.29	2.16	5 W m^{-2}
		amytal	0.51	1.14	1.11	
		rotenone	0.45	0.87	0.93	
Cyt *c* + Apo-ovorubin		-	0.33	0.75	0.84	
		amytal	0.32	0.75	0.84	
		rotenone	0.34	0.66	0.72	
Cyt. *c* + Aged ovorubin	26% reduced	-	-0.42	-0.45	-0.54	
		-				
Cyt. *c* Aged apo-ovorubin		-	-0.45	-0.6	-0.63	
Cyt. *c* + Ovorubin		-	0.42	0.96	1.02	dark
		amytal	0.43	0.9	0.93	
		rotenone	0.4	0.78	0.9	
Cyt. *c* + Apoprotein		-	0.45	0.99	1.02	
		amytal	0.42	0.93	0.96	
		rotenone	0.42	0.75	0.81	
Cyt. *c* + Aged ovorubin		-	-0.45	-0.45	-0.48	
Cyt. *c* + Aged apo-ovorubin		-	-0.42	-0.54	-0.55	

Table 5.3.2

Transfer of reducing equivalents from ovorubin and apo-ovorubin to cytochrome *c* in light and dark

For experimental details see 5.2.3.

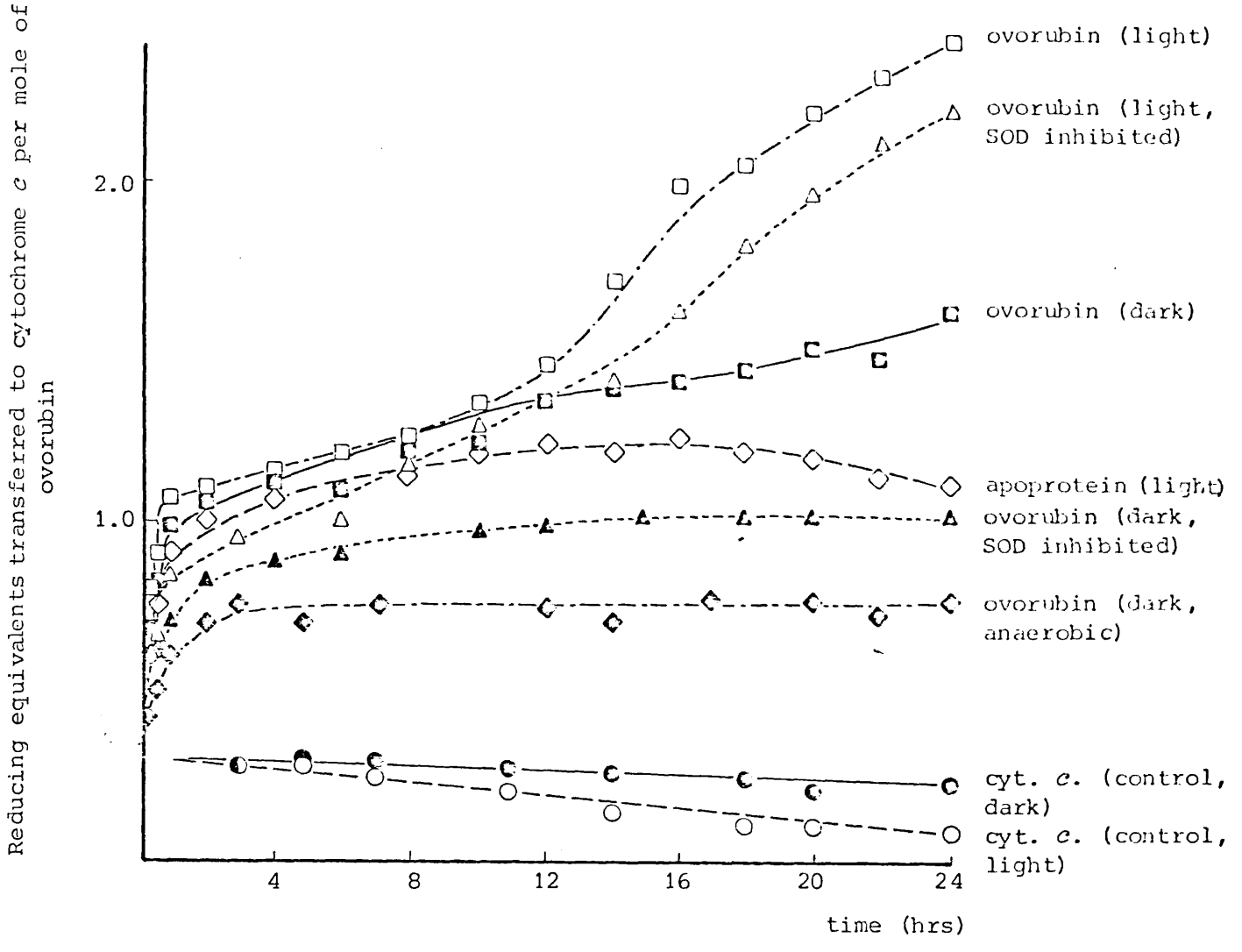

Reducing equivalents transferred to cytochrome c per mole of ovorubin
2.0
1.0
4
8
12
16
20
24
time (hrs)
ovorubin (light)
ovorubin (light, SOD inhibited)
ovorubin (dark)
apoprotein (light)
ovorubin (dark, SOD inhibited)
ovorubin (dark, anaerobic)
cyt. c. (control, dark)
cyt. c. (control, light)

Fig. 5.3.5

5.3.4 The effect of superoxide dismutase on the reduction of cytochrome *c* by ovorubin in the light and dark

Fig. 5.3.5 Reduction of cytochrome (9 x 10^{-6}M) in 0.05M pot. phosphate buffer pH 7.0 at 30°C over a period of 24 hrs by :

- □ ovorubin (3 x 10^{-6}M) irradiated (5W m^{-2})
- △ ovorubin (3 x 10^{-6}M) irradiated (5W m^{-2}) in the presence of 1.2 x 10^{-7} M SOD.
- ◻ ovorubin (3 x 10^{-6}M) dark
- ◺ ovorubin (3 x 10^{-6}M) dark in the presence of 1.2 x 10^{-7} M SOD
- ◇ ovorubin (3 10^{-6}M) dark anaerobically
- ◇ apo-ovorubin (3 x 10^{-6}M) irradiated (5W m^{-2})

The results obtained with the apoprotein of ovorubin in the dark in the presence and absence of 1.2 x 10^{-7} M SOD and under anaerobic conditions are not shown in Fig. 5.3.5 since they closely resemble those obtained with ovorubin. All results shown represent the mean value of three determinations at each point, except for those carried out under anaerobic conditions, which represent a single determination. The experiments were carried out as described in 5.2.4

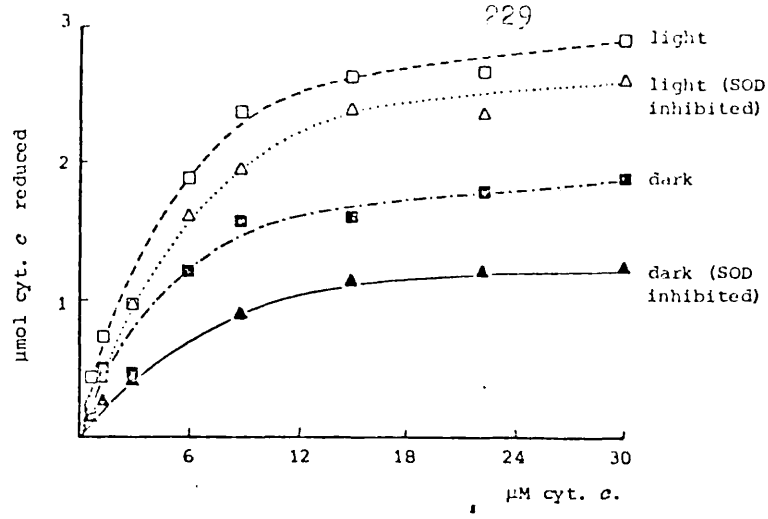


Fig. 3.3.6a

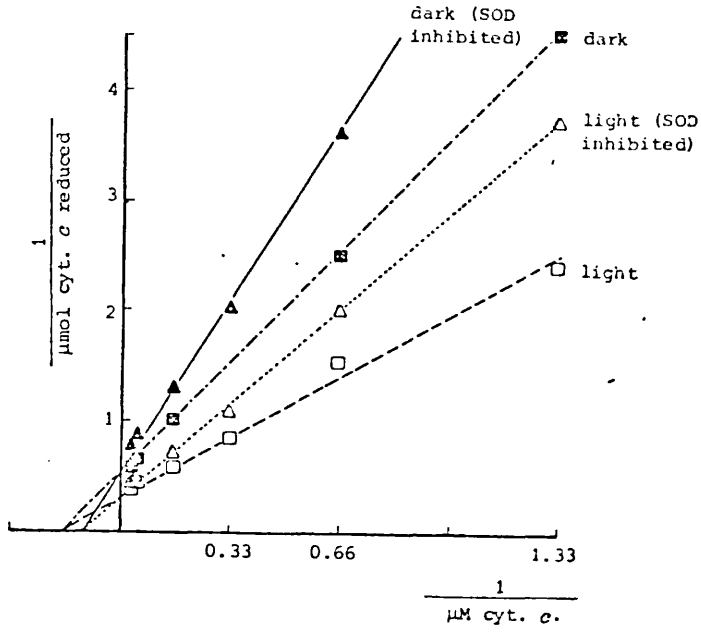


Fig. 3.3.6b

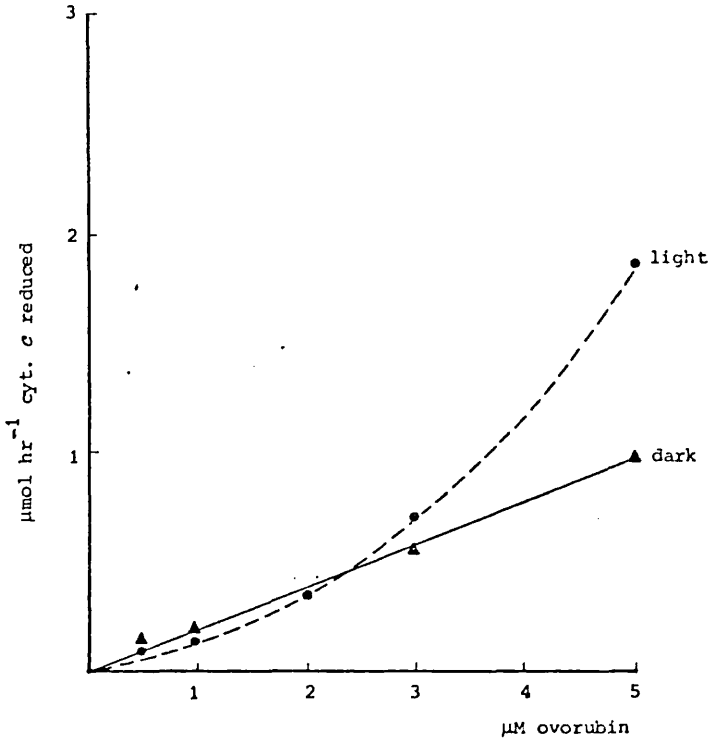


Fig. 3.3.7

5.3.5 The effect of the reactant concentration on the reduction of cytochrome *c*.

Fig. 5.3.6a Effect of cytochrome *c* concentration (0.75-30μM) on its reduction by ovorubin (3 μM) in light (5W m^{-2}) and dark and the inhibition by SOD (1.2×10^{-7}) in 0.05M K phosphate at pH 7.0.

□ light (5W m^{-2})
△ light (5W m^{-2}), SOD-inhibited
■ dark
▲ dark, SOD-inhibited

Measurements were made after 24 hrs at 30°C.

Fig. 5.3.6b Lineweaver-Burk plot obtained from data in Fig. 5.3.6a

Fig. 5.3.7 Effect of ovorubin concentration (0.5 to 5μM) on the reduction of cytochrome (3 x 10^{-6}M) obtained one hr after mixing of the reactants in

• light
▲ dark

The results shown represent the mean values of three determinations.

Experimental details for Fig. 5.3.6a, b and 5.3.7 are given in Chapter 5.2.5.

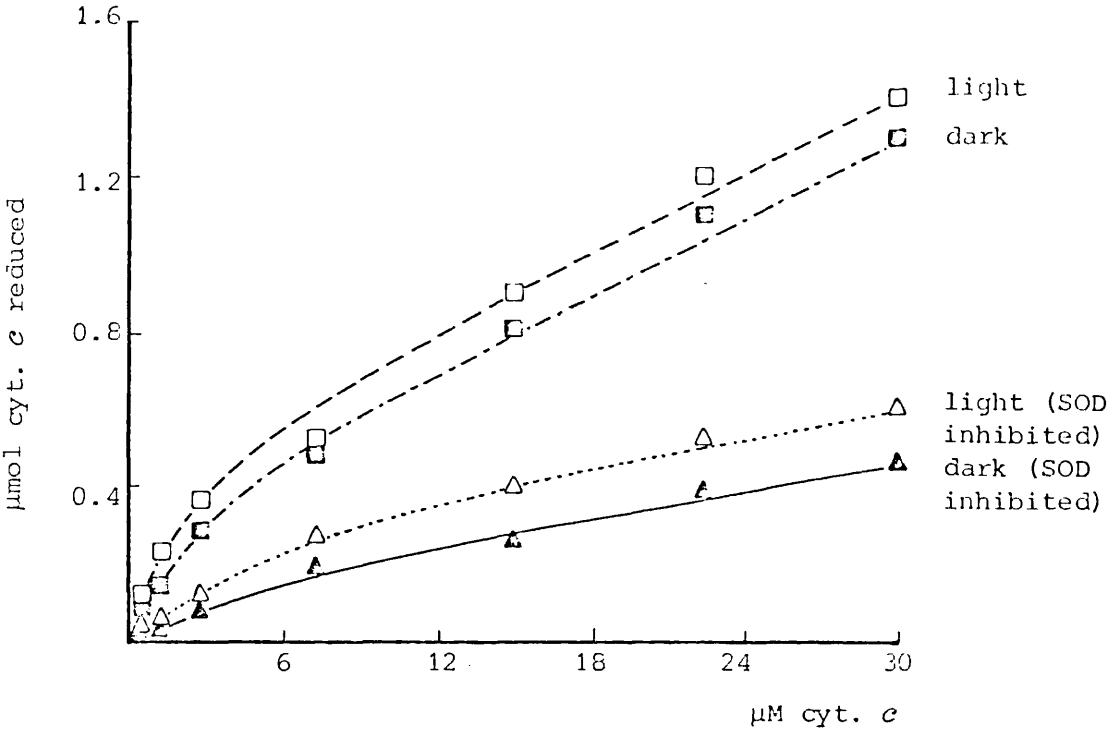


Fig. 5.3.8a

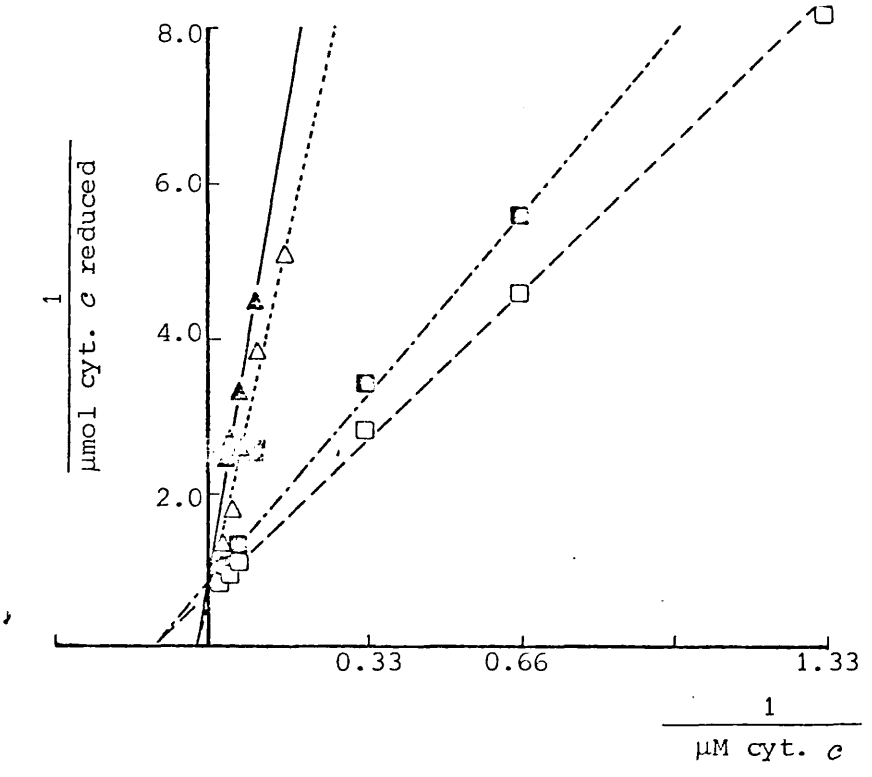


Fig. 5.3.8b

5.3.6 The effect of ageing on the interaction of ovorubin with cytochrome *c*.

Fig. 5.3.8a Reduction of cytochrome *c* (0.75 -30μM) by an "aged" preparation of ovorubin (3 μM) after 24 hrs.

□ light (5 W m^{-2})

□ dark

△ light, inhibited by SOD

△ dark, inhibited by SOD

Fig. 5.3.8b Lineweaver-Burk plot derived from data in Fig. 5.3.8a.

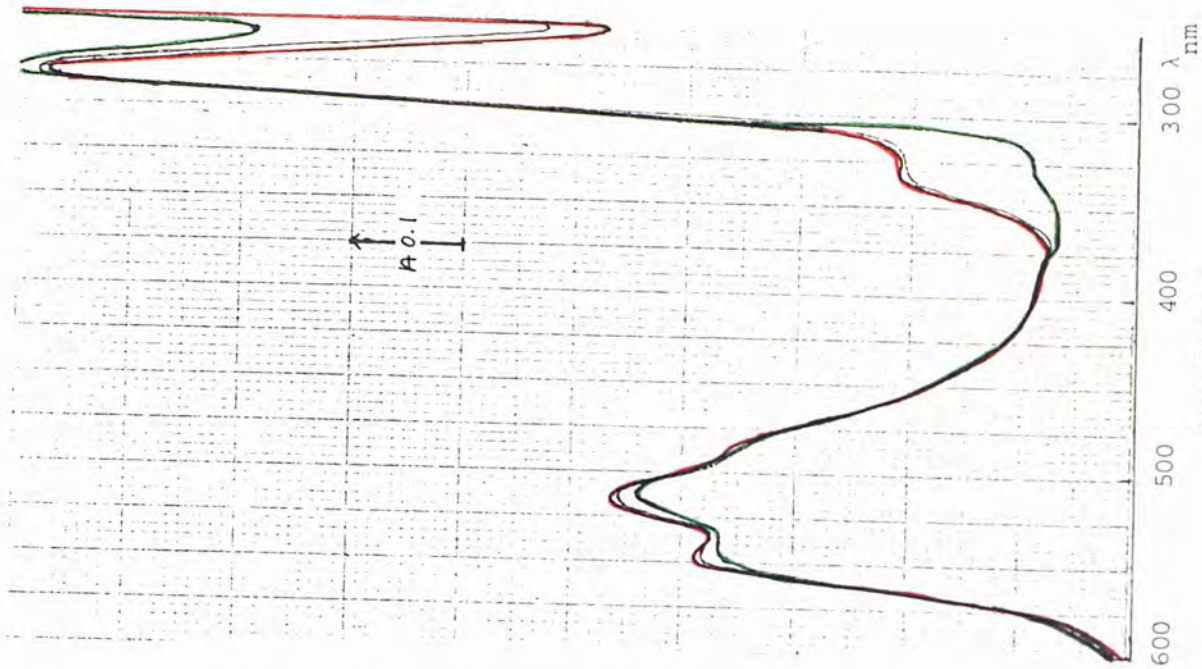


Fig. 5.3.9a

Fig. 5.3.9b

Fig. 5.3.9a The absorption spectrum of ovorubin (approx. 3×10^{-6} M,) five days after purification in O.05M K phosphate buffer pH 7.0

- reduced with 1 mM dithionite
- oxidised with 1 mM Na persulphate
- untreated

Fig. 5.3.9b The absorption spectrum of an "aged" preparation in O.05M K phosphate buffer pH 7.0.

- reduced with 1 mM dithionite
- oxidised with 1 mM Na persulphate
- untreated

For experimental details see 5.2.6

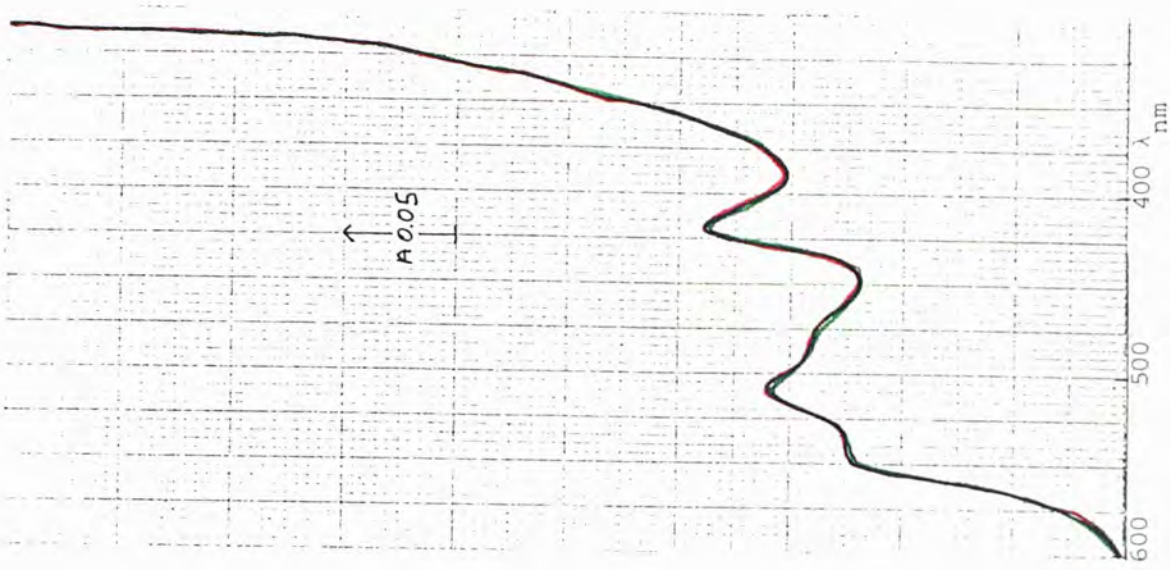


Fig. 5.3.10a

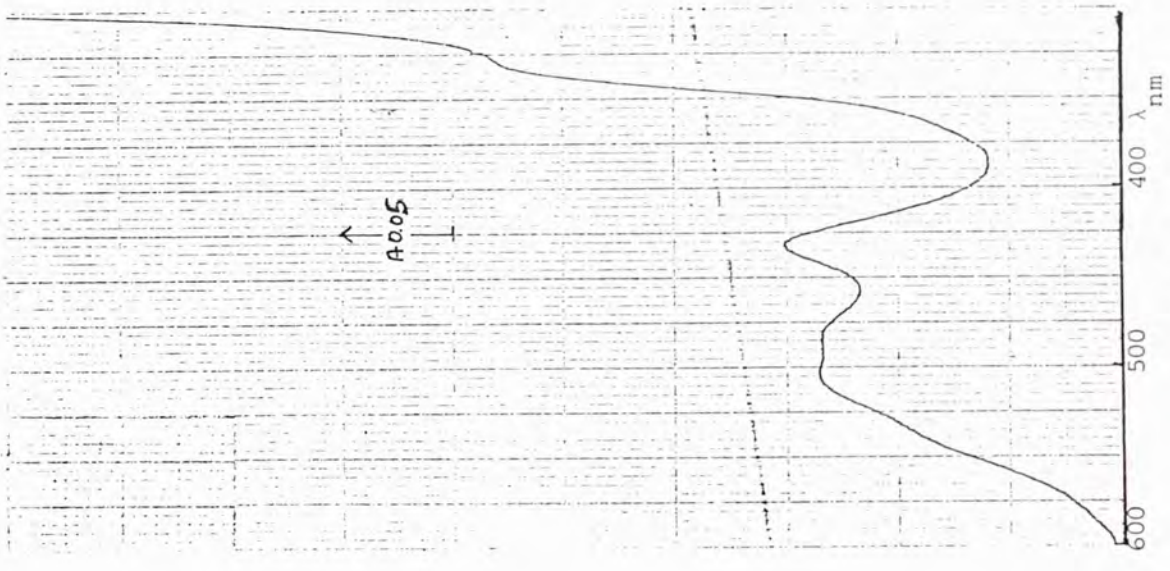


Fig. 5.3.10b

5.3.7 Reisolation of ovorubin after its exposure to cytochrome *c* in the light and in the dark

Fig. 5.3.10a Spectrum of ovorubin reisolated after previous exposure to cytochrome *c* in 0.05 M K phosphate buffer pH 7.0, in the light for 24 hrs.

- reduced with 1 mM dithionite
- oxidised with 1 mM persulphate
- untreated

The spectrum of ovorubin reisolated after previous exposure to cytochrome *c* in the dark was indistinguishable from that shown in Fig. 5.3.10a.

Fig. 5.3.10b Spectrum of the carotenoglycoprotein isolated from the eggs of *Pomacea insularum* in 0.05 M K phosphate buffer, pH 7.0

for expt. details see § 2.7

5.4. Discussion

5.4.1 Reduction of cytochrome *c* by ovorubin in the presence of reducing agents.

The results shown in Chapter 5.3.1 show that ovorubin promotes the reduction of cytochrome *c* by NADH, NADPH, GSH and ascorbate, NADPH and ascorbate being the most effective substrates. The fact that NADH and NADPH have the same V_{max} leads one to suppose that the same catalytic site is concerned in both cases.

The inhibition by amytal and rotenone of the reduction of cytochrome *c* by NADH and NADPH suggest that ovorubin functions like NADH dehydrogenases. These enzymes catalyse the transfer of electrons from NADH and/or NADPH via a flavin prosthetic group to an unknown acceptor which may be a quinone or possibly a non-haem iron protein. A number of flavoproteins have been shown to contain a non-haem iron subunit, although it is not known whether they function as carriers through alternate reduction and oxidation of the iron.

Electronic absorption spectra of ovorubin provide no evidence for the existence of a conventional flavin moiety.

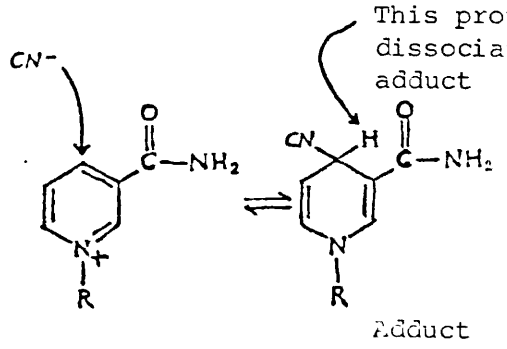
However, e.s.r. spectra measured by Dr. K.D. Sales shows strong signals with g-values of 1.96 and 4.2 suggesting the presence of non-haem iron.

Reduction by GSH and ascorbate is not affected to any significant extent by amytal and rotenone. At present no mechanism can be suggested for the ovorubin-catalysed passage of electrons from these reducing agents to cytochrome *c*. The non-enzymic reduction by GSH is slow, while that by ascorbate is rapid, although increased in the presence of ovorubin.

At first sight, the strong activation of the reduction of cytochrome *c* by NADPH and NADH in the presence of KCN seems rather surprising. In the absence of further detailed investigations of this effect, it may be attributed to the action of one of several factors or a combination thereof:

1. There is the possibility of a weak cytochrome oxidase activity on the ovorubin molecule, which is inhibited by KCN thus leading to an increase in the reduction of cytochrome *c*. No direct evidence for this has been obtained so far, and if such a function were associated with ovorubin, it is normally masked by a stronger cytochrome reductase activity. Very tentative evidence for an oxidase function being associated with a carotenoid moiety come from the work of Karnaukhov (1971) and possibly from the observation that CO-binding to ovorubin leads to spectral changes in the carotenoid region (Fig. 3.3.1c), since O_2^-, CO- and KCN-binding commonly involves identical sites. It appears, however, unlikely that the inhibition of a terminal oxidase function by KCN is responsible for the observed activation, since the reduction of cytochrome *c* by GSH and ascorbate, catalysed by ovorubin, is virtually unaffected by KCN.

2. A number of nucleophilic reagents, including CN^-, add reversibly at the para position of the nicotinamide ring to form adducts with structures reminiscent of the reduced co-enzyme. As a result of the formation of a CN adduct, the portion adjacent to the highly polarised C≡N is easily dissociable.



It appears possible that such an effect may play a role in the observed activation by KCN.

3. Commercial preparations of cytochrome *c* frequently contain trace amounts (0.01%) of cuprozinic SOD, which is inhibited by KCN, thus leading to an increase in the reduction of cytochrome *c* if the $O_2^{\cdot-}$ radical is responsible for, or involved in, this reduction. In conventional assays of the reduction of cytochrome *c* by $O_2^{\cdot-}$, the enhancement of this rate by KCN is taken as a measure of the amount of SOD present (McCord *et al.* 1977).

Experiments by Babior *et al.* (1976) on neutrophile leukocytes show that there exists an enzyme, tentatively identified as an NADPH oxidase, which generates $O_2^{\cdot-}$ in the presence of NADPH and NADH. This activity has been termed a superoxide synthetase by the authors. Results of their studies on substrate specificity are shown below :

Reducing agent	$O_2^{\cdot-}$ production (nmol min^{-1} mg^{-1} protein)
NADPH	5.3
NADH	1.5
GSH	0.05
ascorbate	0.05

For experimental details, see Babior *et al.* (1976). The concentration was 0.1 mM for each reducing agent.

These results indicate that the only reducing agents that the enzyme could use were NADPH and NADH.

Michaelis constants of the enzyme clearly favoured NADPH as the physiological substrate. With NADPH as the reducing agent, the following stoichiometry was obtained :

$$NADPH + 2O_2 \longrightarrow NADP^+ + 2O_2^{\cdot-} + H^+$$

The enzyme is not inhibited by cyanide. If cytochrome *c* employed in the present assays contained trace amounts of SOD, which is inhibited by KCN, this could well explain the observed activation by KCN of the reduction of cytochrome *c* if ovorubin acted in a similar fashion on NADPH and NADH. The observation that the reduction of cytochrome *c* by ovorubin is inhibited by SOD (5.3.4) lends support to this point.

Further experiments investigating the effect of SOD on the reduction of pure cytochrome *c* by NADPH and NADH in the presence of ovorubin, together with measurements of oxygen uptake during the reaction, should help to clarify this point.

5.4.2 Factors influencing the reduction of helicorubin P and cytochrome *c* by ovorubin or its apoprotein in the absence of reducing substrates.

As can be seen from the figures and tables in 5.3.3-6, ovorubin and its apoprotein can transfer electrons to helicorubin P, which can be replaced by cytochrome *c* from *Candida krusei* or horse heart.

This process shows certain distinct features :

1. When ovorubin is added to oxidised helicorubin or cytochrome, the latter is partially reduced in the dark.
2. In visible light, the reduction tends to go to completion.
3. With the carotenoid-free apoprotein, partial reduction occurs in the dark, which corresponds to that obtained with ovorubin under the same conditions.
4. Fig. 5.3.5 shows that the initial phase of the reduction of cytochrome *c* reaches a plateau after 8-10 hours. Closely corresponding levels are obtained with ovorubin or its apoprotein and the degree of reduction is largely uninfluenced by light.

5. In the presence of light, a second phase of reduction occurs, while in the dark there is but a slight increase. The light-stimulated reduction is inhibited by amytal and rotenone (Table 5.3.2).

6. The light-promoted interaction is in some manner dependent on the carotenoid moiety since it is not observed either with ovorubin in the dark, or with the apoprotein in the light.

7. With the apoprotein in the light and to a lesser extent in the dark, the reduction of cytochrome decreases progressively after ca. 14 hrs. This suggests that the carotenoid may protect the cytochrome from light-sensitised oxidation.

8. The reduction of cytochrome *c* can be inhibited partially by SOD, indicating the generation of $O_2^{\cdot-}$ by both ovorubin and apoprotein in the light and the dark.

9. At constant ovorubin concentration, the reduction of cytochrome *c* shows a rectangular hyperbolic saturation curve when its concentration is varied and a typical Lineweaver-Burk plot reminiscent of classical Michaelis-Menten kinetics.

10. Under these conditions, SOD behaves as a competitive inhibitor of the reduction of cytochrome *c* both in the light and in the dark.

11. With freshly prepared ovorubin, this inhibition is more pronounced in the dark than in the light.

12. The ageing of ovorubin greatly diminishes the light-stimulated reduction of cytochrome *c* and the inhibition by SOD in the light very closely approaches that observed in the dark.

In aged preparations, the inflexion at 330-335 nm in the spectrum of ovorubin appears irreversibly altered and is insensitive to oxidising and reducing agents. The loss of absorbance in this region appears to be correlated with increases at 280 and 250-255 nm. Minor changes occur in the absorption bands of the carotenoid.

For comparison of the spectra of a fresh and an aged preparation of ovorubin, see Figs. 5.3.9a and 5.3.9b.

Exposure to light accelerates this ageing process.

13. Very old preparations of ovorubin no longer reduce cytochrome *c*, but produce a slight oxidation of the partially reduced haemoprotein. This process is not affected by light and may be due to a reaction between the cytochrome and the irreversibly altered chromophore.

14. When the concentration of ovorubin is varied at constant cytochrome concentration, a plot of initial velocity of reduction versus ovorubin concentration indicates a first-order process with respect to ovorubin in the dark, and a higher-order reaction in the light.

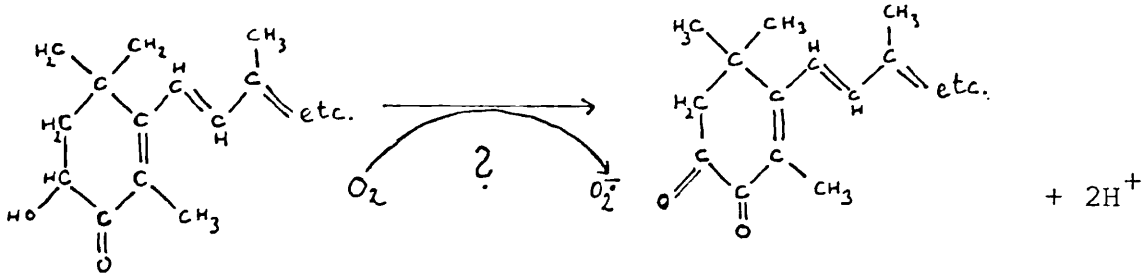
15. Under anaerobic conditions in the dark, the reduction of cytochrome by ovorubin or its apoprotein reaches about one third of the level obtained with ovorubin in the light and about half of that obtained with ovorubin or the apoprotein in the dark.

These observations raise the question of the number and the nature of the groups involved in the reduction of cytochrome *c* by ovorubin and its apoprotein.

The dependence of the second phase of the biphasic response on the presence of both light and the carotenoid prosthetic group, renders it probable that the latter has a function in light-stimulated electron transport; the generation of $O_2^{\cdot-}$ by some centre on ovorubin is clearly responsible for part of the observed reduction of cytochrome *c*. The observation that inhibition of this process by SOD in the light is less prominent than in the dark would suggest either that the light-stimulated reduction does not involve the formation of $O_2^{\cdot-}$, but occurs by some other process, or alternatively that the amount of SOD present is insufficient to deal with the number of radicals produced. In view of the high catalytic efficiency of SOD (McCord *et al.*, 1977), this appears unlikely although the enzyme may have become less effective after prolonged exposure to light at 30°C. The anaerobic reduction of cytochrome *c* by ovorubin in the dark suggests yet a third redox function independent of both light and superoxide. This source of reducing power is exhausted after about two hours, rendering it unlikely that a leakage of oxygen is responsible for the observed effect.

It is tempting to speculate that the small spectral shifts in absorption maxima of ovorubin observed on oxidation and reduction are due to a change in the carotenoid prosthetic group when it passes from the reduced form (astaxanthin) to the oxidised form (astacene).

When removed from the protein binding it, astaxanthin is quite easily oxidised to the diketo form astacene.



The oxidation of astaxanthin to astacene could be associated with the generation of a $O_2^{\cdot-}$ radical in a fashion resembling the formation of superoxide in the oxidation of photoreduced flavin (Massey *et al.* 1969). The observation by Patel and Willson (1973) that quinone derivatives and in particular those substances which share structural similarities with semiquinones, are able to form $O_2^{\cdot-}$ by reaction with oxygen, lends theoretical support to this speculation. As yet, there is no evidence that an astaxanthin/astacene redox couple either exists or has a function in electron transport, in spite of plausible arguments for such a function.

Chromatographic analysis by Zagalsky (personal communication), who isolated the carotenoid fraction of ovorubin 30 min after its interaction with oxidised cytochrome in the light, showed no significant change in comparison with the carotenoid moiety obtained from ovorubin separately. He obtained three, or possibly four fractions, by far the most prominent being astaxanthin.

In view of the lack of inhibition of the light effect by SOD and the observation of Zagalsky, the proposed mechanism of cytochrome *c* reduction via $O_2^{\cdot-}$ formation during the oxidation of astaxanthin to astacene appears doubtful and further detailed studies are required to solve this problem.

An alternative explanation for the light-promoted carotenoid-dependent reduction of cytochrome *c* is suggested by the observation of Rosenberg (1966) who found that when powdered, crystalline ß-carotene was sandwiched between two electrodes and irradiated, an electron current was obtained, the intensity of which was proportional to the incident light. If such a photoconductive process were responsible for the reduction of cytochrome *c*, the transfer of the current must be dependent on the functional integrity of another compound in the chain. This follows from the observation that with aged preparations of ovorubin the light-enhanced reduction of the cytochrome is greatly diminished and the degree of inhibition by SOD in the light approaches that in the dark (Fig. 5.3.8a).

The inhibition of the light-stimulated electron transfer by amytal and rotenone might suggest that a flavin prosthetic group is concerned in this process, although electronic absorption spectra provide no evidence for a conventional flavin moiety.

Identification of the chromophore responsible for the inflexion at 330 to 335 nm in the reduced form of ovorubin, the oxidation of which is accompanied by large changes of absorbance at 250-255 and 280 nm, would greatly aid this discussion which at present can only be speculative. Spectra and difference spectra of both ovorubin and its apoprotein (Fig. 3.3.1a and 3.3.3a) suggest the presence of a quinone or possibly a perturbed bound nicotinamide moiety, rather than a flavin prosthetic group, although the latter is frequently found together with non-haem iron for which evidence has been obtained from e.s.r. studies. This chromophore may well be related to the generation of $O_2^{\cdot-}$ by ovorubin and its apoprotein in both the light and the dark.

The production of $O_2^{\cdot-}$ in biological systems usually appears to involve flavin as generator of the radical. The xanthine oxidase reaction, with xanthine or hypoxanthine as substrate, is a very well characterised source of the $O_2^{\cdot-}$. (McCord and Fridovich, 1969). Massey *et al.* (1969) first reported the production of superoxide by the spontaneous reoxidation of photoreduced flavin in the presence of EDTA, which reduces flavins readily on exposure to

light. In each system, the production of $O_2^{\cdot-}$ is coupled to the reduction of cytochrome *c*.

The reduction of cytochrome *c* by ovorubin and its apoprotein has a number of features in common with systems where the production of superoxide is coupled to the reduction of the haemoprotein. When the concentration of cytochrome *c* is varied, the reduction by $O_2^{\cdot-}$ invariably shows a classical hyperbolic saturation curve (McCord and Fridovich, 1968; Roos, *et al.* 1977). Saturation kinetics are obtained since at low concentration of cytochrome *c*, $O_2^{\cdot-}$ is unable to find cytochromes to accept the electrons, and therefore "auto-oxidises" by way of a dismutation reaction :

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

while at high concentrations complete scavenging of $O_2^{\cdot-}$ by the cytochrome would be approached. The apparent K_m therefore reflects the concentration of cytochrome *c* at which half the superoxide produced would disappear via a second-order dismutation and the other half via a first-order reaction leading to the reduction of cytochrome *c*. Thus, the apparent K_m for cytochrome *c* is a measure of the steady state concentration of superoxide, and therefore of the rate of production of the radical. As a consequence of this, in systems where the enzymic production of $O_2^{\cdot-}$ is coupled to the reduction of ferricytochrome *c*, the K_m for the cytochrome has been found to be a function of the concentration of the enzyme that generates the radical on interaction with O_2. Such a finding is obviously in contrast to the usual binding of a substrate to an enzyme, where the K_m for that substrate is independent of the concentration of enzyme. Such experiments have yet to be performed for the reduction of cytochrome *c* by ovorubin. A positive outcome of such tests would certainly provide further support for a "superoxide synthetase or oxygen reductase" function of ovorubin which as yet is only supported by the fact that the reduction of cytochrome *c* is inhibited by SOD.

This inhibitory protein will intercept the $O_2^{\cdot-}$ radical in free solution thus acting as a competitive inhibitor. This type of inhibition is indeed observed in both the light-stimulated and the dark reaction, although the effect of SOD on the dark reaction or on that catalysed by the apoprotein is far more pronounced.

A plot of initial velocity of reduction versus ovorubin concentration (Fig. 5.3.7) indicates a first-order process with respect to ovorubin in the dark and a reaction of second or higher order in the light. These results suggest that in the light the rate of reaction is proportional either to the square of the concentration of a single reactant or, more probably, to the product of the concentrations of two reactants. In view of the observation that the light-stimulated reduction observed in fresh preparations is dependent on the presence of the carotenoid, it appears reasonable to regard this as further evidence for the participation of the carotenoid in the reductive process. Whether this involvement is a direct one, or whether the carotenoid moiety is required to assist in the maintenance of a conformational feature essential for reduction, cannot be resolved without further experiments. The finding that in aged preparations the carotenoid can no longer support the light-promoted reduction of ferricytochrome *c*, suggests either a coupling mechanism that is destroyed on ageing, a change in binding, or a chemical alteration of the chromophore. There is little or no evidence in favour of an astaxanthin/astacene redox couple which can be reduced in light by a mechanism similar to that invovled in the photoreduction of flavins. The carotenoid moiety in aged preparations may lose activity because of an irreversible change in its mode of combination with the protein. Some support for this can be derived from the spectrum of the carotenoid group *in situ* which in old preparations resembles that observed with the oxidised form of the newly prepared carotenoprotein.

In the dark, the rate of reduction of cytochrome *c* is proportional to the concentration of only one reactant. All the available evidence suggests that the component responsible achieves this by a one-electron reduction of molecular oxygen during its re-oxidation. This is borne out by the fact that this process is competitively inhibited by SOD.

The studies *in vitro* by Patel and Willson (1973) already mentioned, have shown that only those substances capable of yielding strongly reducing semiquinone are capable of reducing molecular oxygen.

A prosthetic group of the flavin type is therefore implied in this reaction, although spectra of the apoprotein do not provide evidence of any known flavin prosthetic group.

It is important to note in this context that the apparent destruction of the chromophore in the 330-335 nm region does not appear to influence the capacity of ovorubin to generate $O_2^{\cdot-}$, although it is associated with the loss of the light-stimulated reduction of cytochrome *c* which is also dependent on the presence of the carotenoid. The possibility must therefore be considered that this chromophore is not involved in $O_2^{\cdot-}$ production, but in the direct transfer of electrons from some other source, perhaps the carotenoid, to cytochrome *c*.

Another question connected with the nature of the chromophore responsible for $O_2^{\cdot-}$ generation is that of the substrate of the reaction, i.e. the agent providing electrons to reduce the redox component which on reoxidation reduces molecular oxygen. Since no substrates have been added to the reaction mixture, it appears most reasonable to assume that another electron donor on the ovorubin molecule provides the reducing power for the component that interacts with O_2 to produce superoxide. Under certain conditions, e.g. in the absence of light, this electron transfer may not be possible, and the component may undergo autooxidation by passing electrons directly to O_2.

It is therefore important to investigate the effect of SOD on the reduction of cytochrome *c* by oxidisable substrates such as NADH and NADPH, which are known to reduce cytochrome *c* in the presence of ovorubin, especially in view of the results discussed in 5.4.1 concerning the activation of the reduction of cytochrome *c* by NAD(P)H in the presence of KCN. The outcome of such experiments should solve the question whether ovorubin possesses a true NAD(P)H-cytochrome reductase activity or whether a NAD(P)H-oxygen reductase function mediated by ovorubin is responsible for the observed reduction of cytochrome *c*.

The answer may provide an alternative explanation for the observation by Cheesman and Helm (1977) who found that a system composed of mammalian lactic dehydrogenase, lactate, NAD, methylene blue and a homogenate of the eggs of *P. canaliculata* shows, in the dark, a weak uptake of oxygen which is greatly increased by visible light. When these homogenates are freed of ovorubin by its absorption onto

Con A-Sepharose, they are no longer effective. Returning ovorubin to the system restores this effect. It was proposed that ovorubin had properties roughly analogous to those of the mitochondrial electron transport complexes I and III. In view of the finding that O_2^- is generated by ovorubin, it is tempting to suggest that the observed uptake of O_2 is related to a promotion by light of this reaction. Although the inhibition by SOD of the light-stimulated reduction of cytochrome *c* observed in the present series of experiments does not lend direct support to such an interpretation, it appears worthwhile to investigate the effect of SOD on the system described above.

The earlier mentioned anaerobic reduction of cytochrome *c* by ovorubin seems to point to the presence of yet another redox carrier.

Somewhat similar observations have been made in studies on xanthine oxidase (Komai *et al.* 1969). On removal of the FAD prosthetic group, the xanthine-cytochrome *c* reductase activity of the deflavo enzyme is independent of O_2. With the holoenzyme, particularly on ageing, a second direct pathway of cytochrome *c* reduction is opened which also functions anaerobically. A non-haem iron prosthetic group is thought to be concerned in this function. Some evidence for such a moiety associated with ovorubin has already been given.

Ovorubin reisolated after its exposure to cytochrome *c* in the light and dark shows a broad absorption peak at ca 418 nm, which cannot be removed by repeated absorption on Con A-Sepharose.

The position of the absorption band at 418 nm is that of the Soret band of reduced cytochrome *c*. This suggests that denatured cytochrome *c* is very firmly bound to the ovorubin molecule, since the position and the intensity of the band is unaffected by oxidising and reducing agents.

Although this is most likely the correct explanation for this observation, a much more speculative interpretation will also be considered. Fig. 5.3.10b shows the spectrum of the carotenoglycoprotein from *P. insularum*, which shares many properties with ovorubin including an astaxanthin prosthetic group and the

chromophore at 330-335 nm (Helm, unpublished observations). It contains yet another unidentified chromophore absorbing at *ca.* 430 nm which is insensitive to oxidation and reduction. The question posed by a comparison between the spectra in Fig. 5.3.10a and b is whether exposure of ovorubin to cytochrome *c* may unmask an oxidised chromophore similar to that associated with the carotenoglycoprotein from *P. insularum* in the native state.

Note added after completion of text :

In the presence of ovorubin, NADH reduces ferricyanide under aerobic conditions (Cheesman, personal communication). If $O_2^{\cdot-}$ is involved in this process, i.e. if ovorubin possesses a NADH-oxygen reductase activity as opposed to a NADH-ferricyanide reductase activity, no reduction of ferricyanide should be observed under anaerobic conditions.

This has indeed been found to be the case. In a simple experiment, ferricyanide was reduced effectively by ovorubin in the presence of NADH; when NADH was added to ferricyanide in a Thunberg cuvette under anaerobic conditions, no reduction was observed until air was allowed to enter the system.

CHAPTER 6.

GENERAL DISCUSSION

The evidence presented in this thesis provides strong support for a functional interaction between the carotenoglycoprotein ovorubin and the cytochrome-like haemoprotein helicorubin P as components of an extracellular, nucleotide-modulated electron transport system.

6.1 Ovorubin and helicorubin P as components of a nucleotide-modulated electron transport system

Results shown in Chapter 2 show that the homogenate possesses oxidising and reducing power which can be released on addition of adenine nucleotides. These are manifested as changes in the redox state of helicorubin following the addition of ATP or ADP to an egg homogenate. Table 2.3 shows that a low Fe^{3+}/Fe^{2+} ratio of the haemoprotein is initially increased with both ATP and ADP, while the converse holds with a high ratio; at intermediate values opposing effects are observed with both ATP and ADP. During the oscillatory oxidation-reduction process that followed the addition of nucleotides, the reductive phase is invariably associated with a decrease in ATP content. Synthesis of ATP during the oxidative phase, however, has only been observed in homogenates of eggs developed for some four days.

On the basis of results shown in Chapter 2 and 5, an electron-transport system is outlined in Fig. 6.1. This is based on the assumption of at least two electron carriers X and Y in ovorubin (OR), which do not interact in the absence of helicorubin (HR). It is proposed that $E^{\circ\prime}_X < E^{\circ\prime}_{HR} < E^{\circ\prime}_Y$. The results described in Chapter 2 suggest that, in egg homogenates, there is a system, closed in the first approximation, where dephosphorylation and phosphorylation related to changes in the Fe^{3+}/Fe^{2+} ratio of the haemoprotein occur and probably enter a state of near equilibrium, analogous to that described by Wilson *et al.* (1974a, b) for mitochondrial oxidative phosphorylation where respiration is dependent on the cytoplasmic $[ATP]/[ADP][Pi]$ ratio = (R_p).

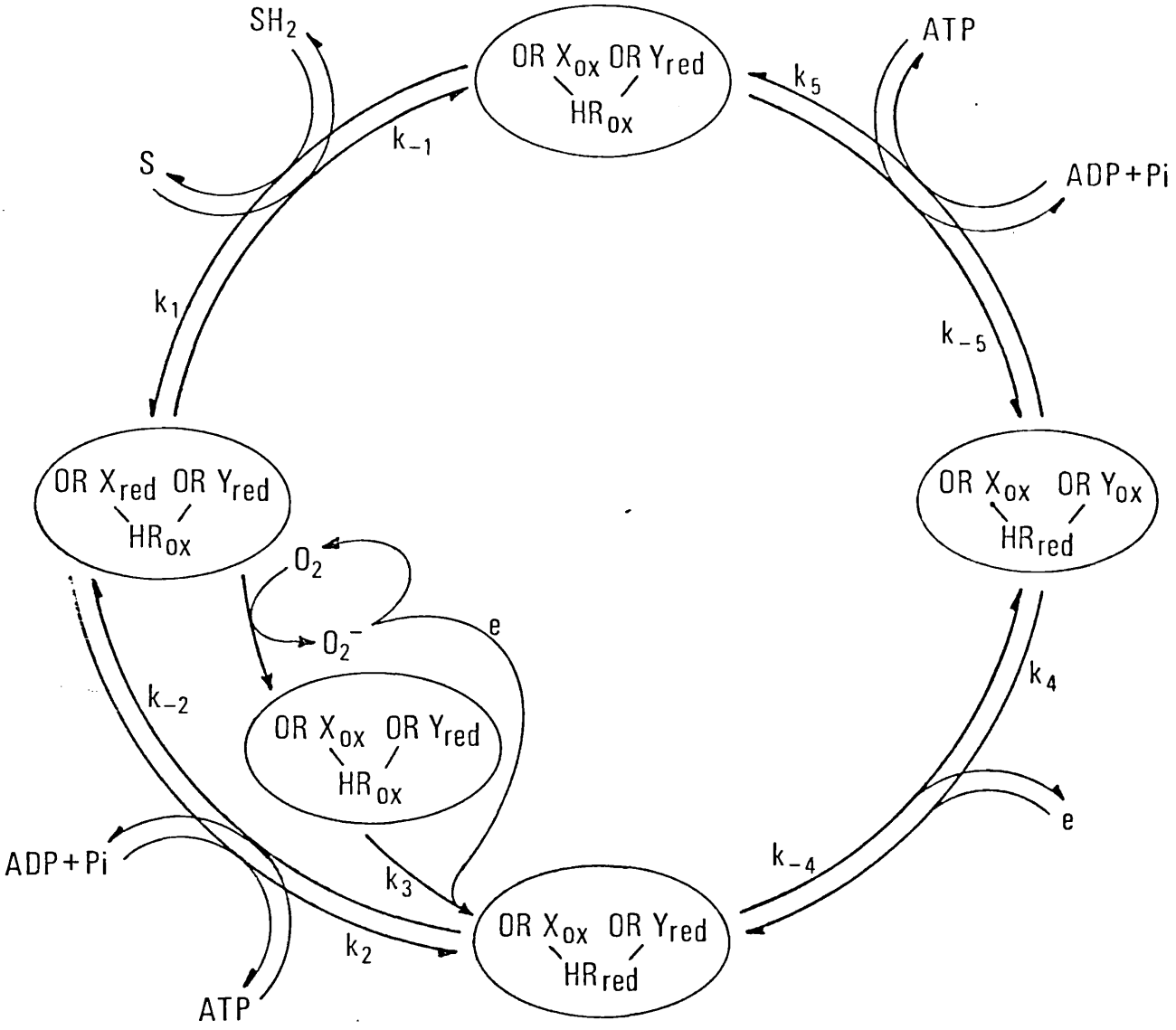


Fig. 6.1.

Hypothetical arrangement of ovorubin and helicorubin P as components of a nucleotide-modulated electron transport system

The reaction pathway outlined in Fig. 6.1. appears consistent with the following picture:

a) At intermediate or high Fe^{3+}/Fe^{2+} ratios, the initial reduction of HR is invariably associated with a decrease in the ATP level of the homogenate. Events associated with this would be governed by K_{-5}, the reaction constant of reaction 5.

b) Under appropriate conditions (see below) the reversal of this reaction, associated with the oxidation of HR, will lead to ATP synthesis, a phenomenon which has been observed in homogenates of eggs incubated at 30°C for some 100 hrs after laying.

c) At low Fe^{3+}/Fe^{2+} ratios of HR, high levels of either ATP or ADP lead to an increase in this ratio. It is tempting to speculate that this process (reaction 2), which is considered to bring about an increase in X_{red}, also involves reductive dephosphorylation, although no evidence can be given for or against such a proposal. If this were so, one might expect that the reversal of this step, involving synthesis of ATP, could occur in a fashion similar to that proposed under b. In each case, the span of redox potential between HR and X need not be more than 200-300 mV.

d) Reduction of helicorubin by X_{red} is thought to occur as follows:

   i) In the presence of endogenous substrate, the route governed by K_3 is predominantly utilised, involving the oxygen reductase activity of ovorubin. This step, when inhibited by anaerobiosis and SOD, and possibly also by CO and N_3^-, leads to oxidation of the haemoprotein if $K_2 \ll K_3$ and the availability of oxidisable substrate is rate-limiting.

   ii) In the presence of high concentrations of substrate both routes K_2 and K_3 are utilised for the reduction of helicorubin.

iii) Competition for electron acceptor (i.e. HR) may lead to inhibition of either pathway by the operation of the other. This would account for the fact that KCN enhances the reduction of helicorubin in the presence of exogenous NAD(P)H, GSH or ascorbate.

iv) The reduction of helicorubin via the pathway governed by K_2 is inhibited by amytal and rotenone, and the presence of these inhibitors, in the absence of exogenous substrate, will therefore enhance the oxygen reductase activity of ovorubin.

The effects of ATP on the degree of reduction of helicorubin have been explained on the basis of reductive dephosphorylation. It appears likely, however, that some of the analogous effects of ADP may be traced to the presence of small quantities of ATP in commercial preparations. The overall effect of adenine nucleotides on the system appear to be due to a change in R_p. It seems probable that $[HR\ Fe^{3+}]/[HR\ Fe^{2+}]$ is either proportional to $[ATP]/[ADP]\ [Pi]$, or is at least profoundly influenced by it.

The question of the ultimate oxidant, or the factor responsible for the oxidation of Y_{red} in ovorubin, is at present unresolved and requires further attention. Cheesman observed a small uptake of O_2 in Warburg experiments on egg homogenates. He also noticed the apparent liberation of O_2 on addition of tentative substrates and found that the gas is very slowly utilised. Evidence described in Chapter 2 provides only circumstantial evidence for the existence of a particulate cytochrome oxidase.

It is evident that the electron transport system in the egg homogenate must be of greater complexity than that outlined in Fig. 6.1. Experiments described in Chapter 5 indicate the involvement of possibly two or three different redox functions of ovorubin, represented by the different pathways for the reduction of helicorubin as summarised in Fig. 6.2.

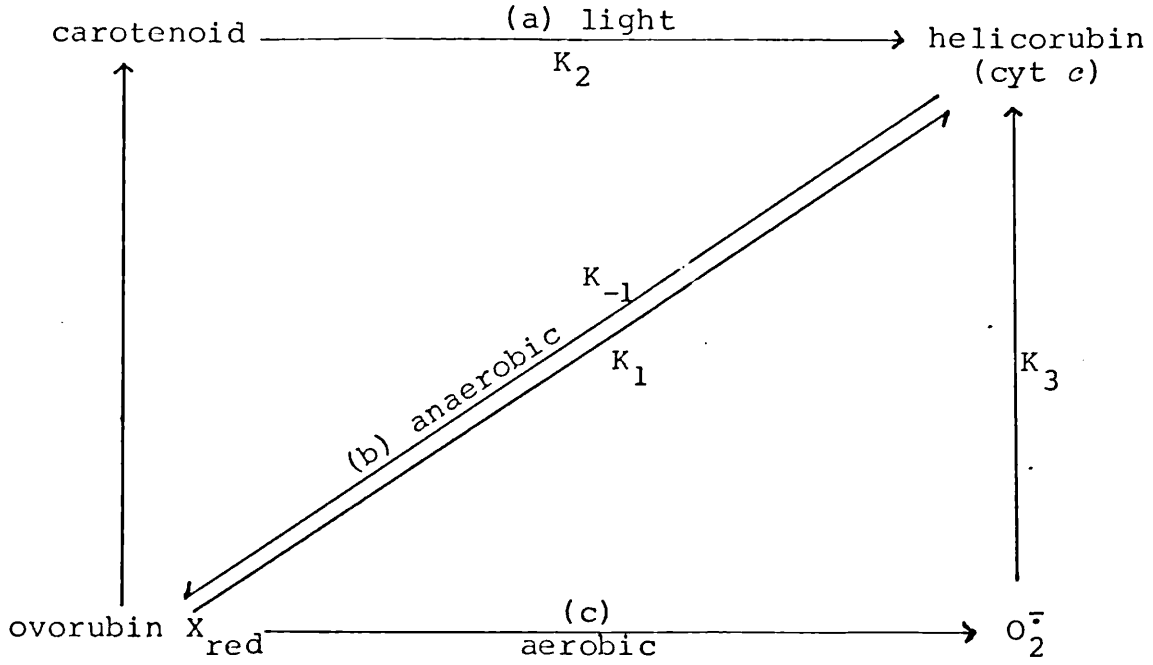


Fig. 6.2. Pathways for the reduction of helicorubin

The pathways involved in the reduction of the haemoprotein under different experimental conditions may be as follows:

			Pathway for the reduction of helicorubin
1.	Ovorubin	light	a + b + c
2.	"	dark	(a) + b + c
3.	"	light + SOD	a + b + (c)
4.	"	dark + SOD	(a) + b + (c)
5.	"	dark (anaerobic)	(a) b
6.	"	dark + SOD (anaerobic)	b
7.	Apoprotein	light	b + c
8.	"	dark	b + c

The simplest explanation for the sigmoidal curves observed in 1 and 3 (see Fig. 5.3.5.) is that partial oxidation of X_{red} (which may comprise two or more redox centres) results in a configurational change in the ovorubin complex. Thus the transfer of electrons from X either to a centre which owes its properties to the presence of the carotenoid, or to the carotenoid itself, with subsequent transfer to the haemoprotein, may be facilitated.

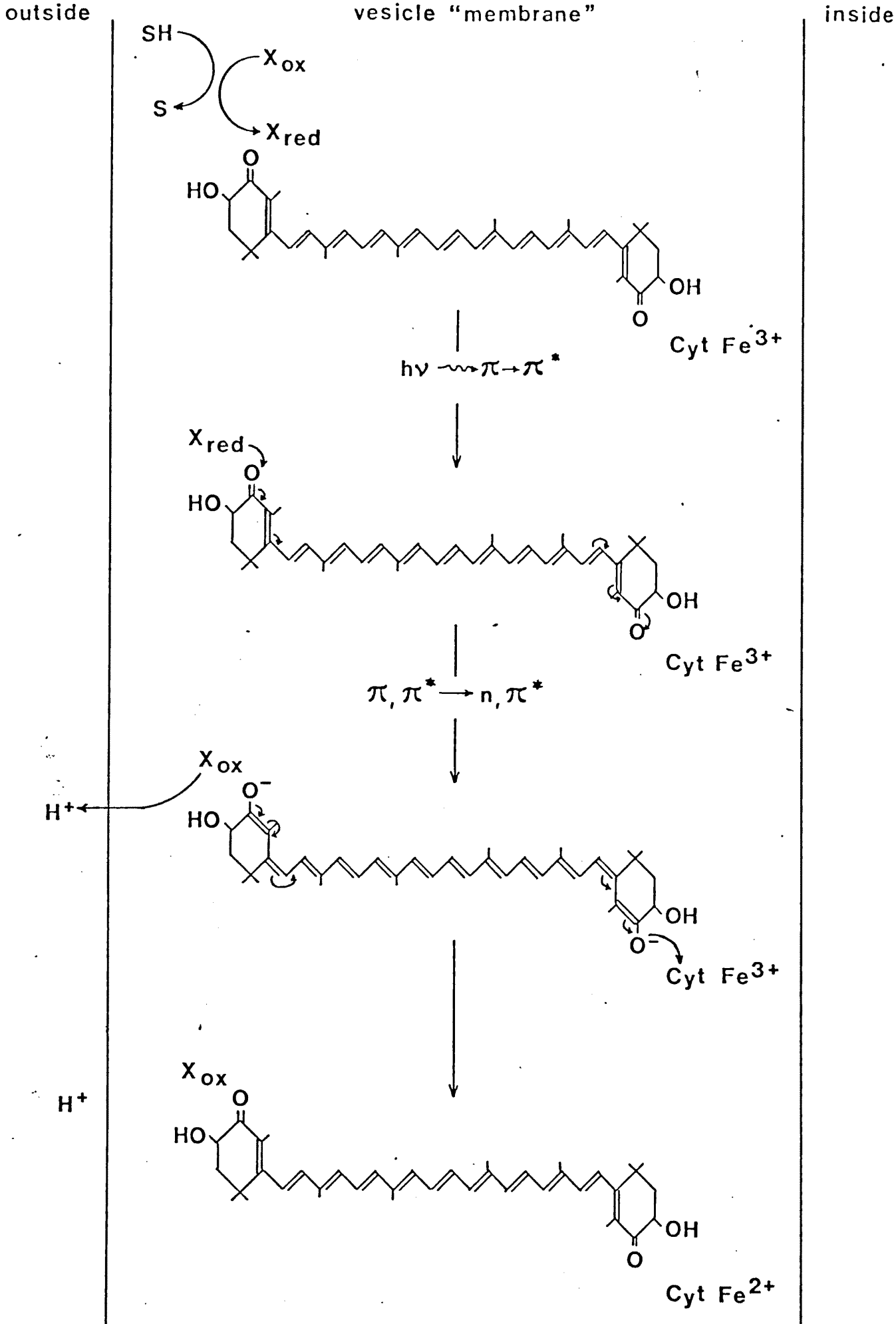


Fig. 6.3

Hypothetical mechanism for the light-promoted reduction of helicorubin by ovorubin

6.2. The effect of light on the reduction of helicorubin or cytochrome *c* by ovorubin

Experiments described in Chapter 5 showed that the light-promoted reduction of the haemoprotein by ovorubin is dependent on the carotenoid moiety, since it is not observed with either ovorubin in the dark, or with the apoprotein in the light.

A molecule, in order to make efficient use of available light energy, must have a system of relatively loosely bound π electrons, which can be raised to the excited state. Light with its electric vector in the plane of a molecule containing a series of conjugated double bonds, will have a high probability of exciting a $\pi \rightarrow \pi^*$ transition. Experiments by Rosenberg (1966) suggest that carotenoid is excited by light to a triplet state, which becomes a kinetic intermediate in charge carrier production. A C=O substitute to a molecule containing a series of conjugated double bonds will involve both a "lone pair" (n-orbital) as well as overlapping or π bond orbitals.

If we examine the energy states associated with such orbital promotions, a number of possibilities present themselves:

1. One of the lone pair electrons is excited to a π^* orbital leaving a "hole" in the n orbital.
2. One of the π electrons is excited to a π^* orbital. Experimentally, however, $n \longrightarrow \pi^*$ transitions are only observed as long wavelength shoulders on the main transitions, indicating a very large overlap in energy levels.
3. It might hence be expected that subsequent to $\pi \rightarrow \pi^*$ promotion, the lone pair may "jump" into the ground π system, which corresponds to a $\pi,\pi^* \rightarrow n,\pi^*$ transition.

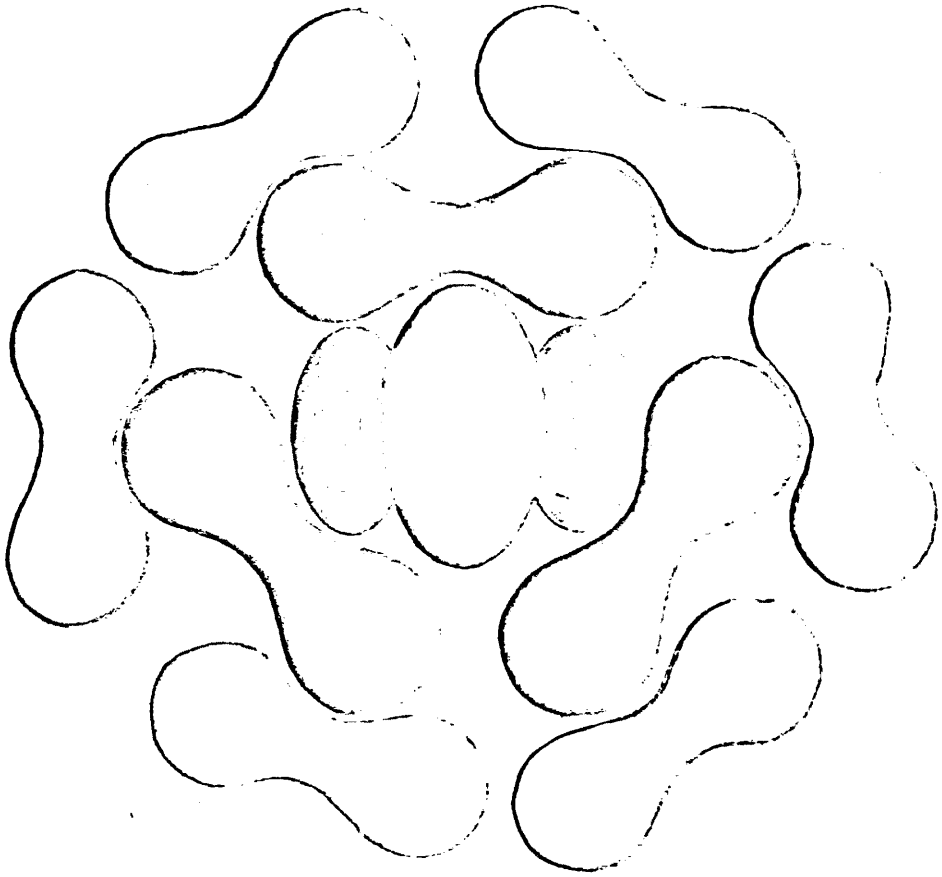


Fig. 6.4

Molecular model for the ovorubin molecule

This "hole" left by the excitation of electrons, could then be filled with an electron supplied from a neighbouring molecule in the chain.

Thus, the exciton, produced by absorption of a quantum, will migrate until it meets an oxidising centre. It is suggested that excitation by light of the keto-carotenoid of ovorubin may be coupled to the reduction of the haemoprotein in a manner outlined in Fig. 6.3.

It is known that in the absence of oxygen, triplet-triplet energy transfer is very efficient, while oxygen in the ground state quenches the triplet excited state of an organic molecule. If the carotenoid were buried inside the protein molecule in a hydrophobic pocket, the haem group of helicorubin P might be aligned in such a way to the carotenoid moiety of ovorubin that electron transport could proceed. It is tempting to speculate that helicorubin P has evolved as a basic protein, in contrast to helicorubin from *Helix*, which is an acidic cytochrome, in order to achieve close complementary alignment with the acidic carotenoprotein.

6.3. The molecular organisation of ovorubin

Electrophoretic analysis shows that ovorubin gives rise to four different sub-units which are present in a 1:2:3:6 ratio. Electron micrographs of the purified protein indicate that most of its sub-units are arranged in two concentric hexagons with two or three subunits occupying a central position.

It was suggested in Chapter 3 that the innermost sub-unit, S_3, is involved in carotenoid binding and accompanied by two sub-units of the S_4 type. This central complex is thought to be surrounded by S_1 sub-units, which according to Cheesman may have a dumbbell shape and therefore appear as pairs of globules in electron micrographs. Three such pairs may constitute the inner hexagon, while similarly dumbbell-shaped sub-units may form the outer hexagon. These are thought to be six sub-units of the S_2 type. Fig. 6.4. shows a diagram of this arrangement.

6.4. The mechanism involved in ATP synthesis

Experiments described in Chapter 2 clearly indicate that the presence of ovorubin and helicorubin alone does not suffice for ATP synthesis and that an additional factor or factors, yet to be identified, are involved. However, when observed, phosphorylation seemed to be dependent on the presence of ovorubin, although its return to homogenates freed from it does not restore the reaction.

The finding that reconsituted systems and homogenates centrifuged at 85,000g no longer support ATP synthesis or show oscillatory behaviour suggests that the two processes are linked and may involve a common component.

The most likely explanation seems to be that either a coupling factor co-purifies with ovorubin on Con A-Sepharose, but has a higher affinity for the lectin and is therefore retained, or that a particular spatial conformation is required for the process, which is disrupted on removal of ovorubin or during high-speed centrifugation.

It appears unlikely that the ATP-dephosphorylating enzyme found in homogenates of *P. canaliculata* eggs performs a function analogous to that of "energy-transducing" ATPases in mitochondrial, chloroplast and bacterial membranes. Although these ATPases are found in widely different organisms and organelles, they are very similar with regard to structure and kinetic properties, which points to a common evolutionary origin. In contrast to this, the magnitude of the K_m for ATP, the lack of substrate specificity and the product inhibition observed with the enzyme from *P. canaliculata* eggs, clearly indicate that we are dealing with a different class of enzyme.

On the other hand, energy conservation may not be dependent on the presence of an energy-transducing ATPase, but involve a mechanism analogous to that found in halobacteria, where a light-driven hydrogen ion pump is coupled to ATP synthesis.

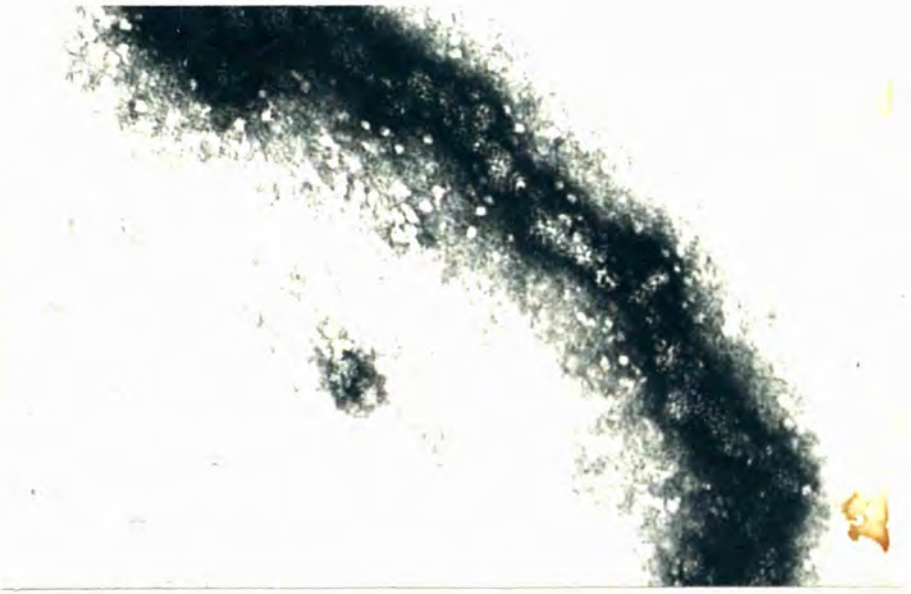


Fig. 6.5
Electron micrograph of an apparently aggregated form of ovorubin.
Magnification: 180,000

Electron micrographs and electrophoretic analysis of ovorubin indicate a tendency for formation of aggregates (Table 3.3.2 and Fig. 6.5). It is therefore conceivable that the electron transport system comprising ovorubin and helicorubin is capable of spontaneous vesicle formation. In such a way, a "proton pump" operated by electron flow may be linked to phosphorylation of ATP in a fashion similar to that observed in halobacteria where light-induced proton translocation is coupled to ATP synthesis. This would be consistent with Mitchell's hypothesis (Mitchell, 1966) that it is the function of the electron carriers to serve as an active transport system or "pump" to transport H^+ ions across the membrane. The electrochemical gradient thus generated would then drive the synthesis of ATP by causing the dehydration of ADP and Pi.

A provision for hydrogen ion translocation is made in Fig. 6.3.

6.5. Developmental aspects

Although the effects of adenine nucleotides on the redox state of helicorubin are observed with freshly laid eggs, linked synthesis of ATP is only observed with partially developed eggs. A number of possibilities must therefore be taken into consideration:

a) a contribution from mitochondria or cytosomes derived from the developing embryo;

b) emergence of substrate level phosphorylation;

c) a slow, spontaneous assembly of the haemoprotein and the carotenoglycoprotein into an electron-transport system coupled to ATP synthesis:,

d) activation of the electron transport system involving ovorubin and helicorubin by an organiser secreted from the ovum or the blastomeres.

Although it is improbable that mitochondria would survive the hypotonic conditions of the experiment, this cannot be ruled out at the present stage of the investigation. The same might apply to cytosomes. It is, however, conceivable that, if such organelles were present, they would still be found in

supernatants of 35,000 g homogenates since they appear to have a higher lipid content than mitochondria, which is expected to lead to decrease in their buoyant density.

A further contribution to the increase in ATP levels could be brought about by substrate level phosphorylation, since Cheesman (unpublished observations) has obtained some evidence for the presence of a glycolytic system in egg homogenates. It is, however, difficult to understand why ATP synthesis by either process should be closely related to the change in the degree of reduction of helicorubin.

The two later possibilities (c and d) therefore seem to provide a more likely explanation for the observed link between the level of ATP and the redox state of helicorubin in egg homogenates from partially developed eggs. The change in the appearance of the egg jelly from an opaque pink to a more translucent red within one to two days of laying was observed by Hylton-Scott (1934), who found that environmental factors such as light and temperature influenced the speed with which these changes took place.

Further experiments should show whether there is a correlation between these changes and the biochemical properties of the eggs, and whether the change in the properties of the electron transport system involving ovorubin and helicorubin is a slow spontaneous event or is affected by some substance released from the developing embryo.

6.6. Functional aspects

The egg jelly of molluscs has commonly been regarded as an inert and undifferentiated fluid serving solely as a nutrient medium for the embryo. The presence of an electron transport system, the major components of which are excluded on quantitative grounds as part of the ovum - the ovorubin content of an egg is at least 5,000 times the weight of the ovum - raises a number of questions:

Is the presence of such a system of physiological significance in the egg jelly? If this is so, what is its function? Since, in their native environment, the eggs are laid in places exposed to intensive solar radiation, water conservation is of obvious importance, and it is possible that the system is geared to the active retention of water, although water movement is usually regarded as secondary to the energised secretion of ions accompanying it (Widdas, personal communication).

Alternatively, the system may only function in the embryo, where ovorubin and helicorubin are found in the digestive gland. It would be important to determine whether the electron transport proteins are transferred to the embryo as a functional unit, or broken down and resynthesised. If the former alternative could be demonstrated, such a massive transfer of material of maternal origin would open new perspectives in chemical embryology.

6.7. Generation of superoxide and the protective function of the carotenoid

The photoprotective function of carotenoids in aerobic systems has been accepted for over 20 years. Results given in Chapter 5 (Fig. 5.3.5.) show that the reduction of cytochromes by the apoprotein decreases progressively when the system is exposed to light. A similar observation is made with the reduced cytochrome when it is exposed to light, but the effect is absent in the presence of ovorubin in light, and somewhat diminished with the apoprotein in the dark.

Evidence presented in Chapter 5 shows that ovorubin catalyses the univalent reduction of O_2, which is inferred from the inhibition by SOD of the reduction of cytochrome by ovorubin. Although there is little evidence that $O_2^{\cdot-}$ is toxic, the deleterious effects associated with the production of this radical are thought to be associated with its alleged tendency to form $OH^{\cdot}$ and singlet oxygen. Since such effects are

usually attenuated by superoxide dismutases, it would be interesting to look for such an enzyme in egg homogenates from *P. canaliculata* . One might, however, expect that, even in the absence of a SOD, the carotenoid would provide some protection in dealing with singlet oxygen potentially formed from O_2^-.

It hence seems reasonable to propose that in systems where O_2^- is generated, carotenoid will not only provide protection against photodynamic action, but may generally attenuate the toxic effects of O_2 in aerobic systems.

6.8. Plans for future work

Observations on the nature and interaction of the carotenoglycoprotein ovorubin and the haemoprotein helicorubin P have shown that these two proteins form part of an electron transport system. The following topics suggest themselves for further study:

1. It could be interesting to establish a possible function for this system and to determine:
   a) whether it is involved in oxidative processes in the extracellular medium;
   b) whether it is transferred as a functional unit into the embryo during development;
   c) whether it is activated by some substance secreted by the ovum or blastomeres;
   d) whether it involves components other than those that have been our concern.
2. The mechanism involved in the synthesis of ATP, which appears to be intricately connected with points mentioned under 1.
3. Further resolution of the system into well-characterised components in order to determine the manner in which they interact.

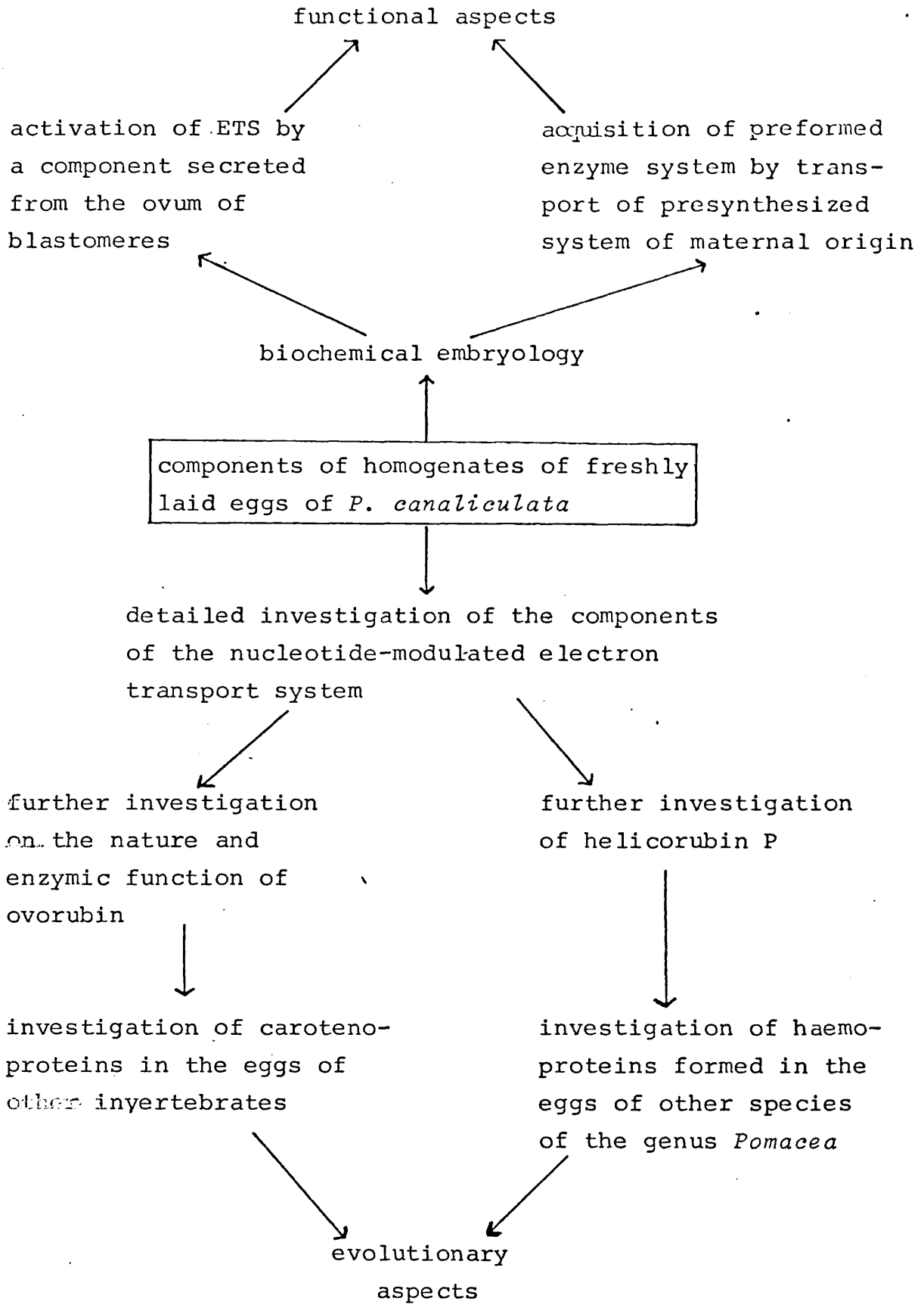


Fig. 6.6

Plans for future work

4. The detailed structure of the apparently polyfunctional protein ovorubin.

5. The possible function of the carotenoid prosthetic group. The occurrence of carotenoproteins in the eggs of invertebrates has been commonly reported (Cheesman *et al.* 1967), although their function is obscure.

6. Since, in the genus *Pomacea*, all eggs investigated showed the presence of two chromoproteins, one of which is a haemoprotein, the possibility of their interacting in a fashion similar to that observed in *P. canaliculata* may be interesting from a taxonomic point of view.

NOTE ADDED AFTER COMPLETION OF THESIS

In Chapter 5 evidence is given that ovorubin has oxygen reductase activity which leads to the generation of the superoxide radical. During its dismutation reaction in aqueous solutions, this ion produces chemiluminescence. Results by Cheson *et al.* (1976) showed a peak between 560 and 580 nm in the spectrum of light emitted by $O_2^{\cdot-}$-producing granulocytes. Emission at such a wavelength, due to the presence of ovorubin, could lead to an amplification of firefly luminescence in response to ATP, which is measured at 562 nm in the Aminco Chem Glow photometer.

Preliminary experiments indeed show that this is the case. Addition of 2×10^{-7} M ovorubin to a firefly extract produced a deflexion corresponding to the 50 pmol ATP standard. Addition of both 2×10^{-7} M ovorubin and cytochrome *c* showed some 20% decrease in the level of chemiluminescence obtained with ovorubin only.

These results suggest that the haemoprotein may scavenge part of the $O_2^{\cdot-}$ formed, while most of the superoxide will "autoxidise" via its dismutation reaction.

It is likely that the substrate for the generation of superoxide by ovorubin is found in the crude firefly lantern extract.

These observations render the measurements of ATP Concentrations in the homogenate unreliable, since they may not reflect the true level of nucleotide owing to chemiluminescence produced during the dismutation of the $O_2^{\cdot-}$ radical. The situation is further complicated by the presence of the haemoprotein, the reactivity of which with $O_2^{\cdot-}$ is influenced by its degree of reduction.

Although the conditions leading to the production of $O_2^{\cdot-}$ are unknown, it appears improbable that the apparent change in the level of ATP described in Chapter 2 can be related to the fate of $O_2^{\cdot-}$. This is borne out by the fact that changes in the degree of reduction of helicorubin are frequently observed without concomitant changes in the apparent ATP levels.

A further possible, although unlikely, source of error might arise from the competition for oxygen, which is utilised in the

generation of superoxide and in the luciferase reaction. Superoxide is known to produce a chemiluminescence of much greater intensity by oxidation of luminol or *Pholad* luciferin (Henry and Michelson, 1977). This is, however, unlikely to happen with firefly lantern luciferin, where light emission is initiated by ATP only.

SUMMARY

The investigation has been concerned with the molecular properties and the functional interaction of the two major proteins in the egg jelly of the South American prosobranch mollusc *Pomacea canaliculata australis* (d'Orbigny). These proteins are:

i) A carotenoglycoprotein, ovorubin (Cheesman, 1954, 1956, 1958) of molecular weight in the region of 300,000. Present investigations indicate that *ca.* 50% of the total protein in the egg is ovorubin.

ii) A haemoprotein, spectroscopically indistinguishable from the helicorubin of *Helix pomatia* (Keilin 1956, 1957, 1968) which represents a further 7-8% of the total protein.

The experimental work on this protein system described in the present thesis has had the following outcome:

1. Ovorubin and helicorubin occur in freshly laid eggs in an approximately 1:1 molar ratio. On quantitative grounds, it is excluded that the above-mentioned proteins are exclusively in the ovum (Hylton Scott, 1934), since the ovorubin content of an egg is at least 5×10^3 times the weight of the ovum.

2. Ovorubin and helicorubin form part of an extracellular electron transport system. Helicorubin in the homogenate becomes further reduced on addition of NADH, NADPH, GSH and ascorbate. This reduction is inhibited in varying degrees by amytal, rotenone, heptylquinoline oxide, antimycin and oligomycin, but enhanced by azide and KCN. In the absence of added reductants, the former group of inhibitors increase the reduction of the haemoprotein, while the latter cause oxidation, an effect also observed with carbon monoxide.

3. The redox state of the cytochrome is changed on addition of ATP, ADP and AMP. The response obtained can be related to the initial degree of reduction of the haemoprotein and is dependent on the concentration of the nucleotide. In every case these initial effects represent the opening phase of a slow oscillatory phenomenon, during which helicorubin is

alternately reduced and oxidised or vice versa. Homogenates in which no ovorubin can be detected spectroscopically after its adsorption on Con A-Sepharose, show greatly reduced sensitivity to adenine nucleotides. When ovorubin is returned to the system, this sensitivity is largely restored, although the oscillatory phenomenon is lost.

4. The reoxidative phase after addition of ADP + Pi may be associated with ATP synthesis, while, during the reductive phase, there is a disappearance of ATP from the homogenate. In homogenates of freshly laid eggs, no ATP synthesis is obtained. Eggs, developed for over 100 hrs, begin to show ATP synthesis, which is associated with redox changes in the cytochrome. This is not observed in homogenates freed from ovorubin.

5. Egg homogenates catalyse the dephosphorylation of both ATP and ADP. About 10% of this activity co-purifies with ovorubin in chromatography on Con A-Sepharose gels. Further purification of ovorubin by micropreparative electrophoresis leads to a complete loss of the adenine nucleotide dephosphorylating activity.

6. Ovorubin has been resolved by SDS-PAGE into a well-defined sub-unit structure. On the basis of these results and electron micrographs of the protein, a molecular model of the protein is proposed.

7. Ovorubin shows characteristic spectral changes on exposure to CO. The spectrum of the apoprotein is not affected by CO. The inflexion at 330 nm in the absorption spectrum of ovorubin previously thought to be associated with the carotenoid (Cheesman, 1958), has been shown to be a feature of the apoprotein and is sensitive to oxidation and reduction.

8. E.s.r. spectra of ovorubin show signals at g-values of 1.96 and 4.2 suggesting athe presence of non-haem iron.

9. Helicorubin from *P. canaliculata* has been purified and shown to be a basic protein, in contrast to helicorubin from *Helix pomatia*, which is an acidic cytochrome.

10. Studies on the isolated protein show that ovorubin catalyses the reduction of helicorubin by NADH, NADPH, ascorbate and GSH. In these reactions helicorubin may be replaced by cytochrome *c* from horse heart or from the yeast *Candida krusei*. KCN is a potent activator of the reduction in the presence of NADH and NADPH, while amytal and rotenone inhibit the reaction.

11. When ovorubin is added to its accompanying haemoprotein helicorubin or cytochrome *c* in the oxidised form, partial reduction occurs in the dark. In visible light, the reduction is virtually complete. With the carotenoid-free apoprotein, partial reduction occurs, either in the light, or in the dark, to the same degree as that found with the carotenoprotein in the dark. The initial phase of the reduction of the cytochrome reaches a plateau after 8-10 hrs. Closely corresponding levels are obtained with ovorubin and its apoprotein; the degree of reduction is largely uninfluenced by light. In the presence of light, a second phase of reduction occurs, while in the dark there is but a slight increase. The light-stimulated reduction is inhibited by amytal and rotenone. The light-promoted interaction appears to be in some way dependent on the carotenoid moiety, since it is not observed with either ovorubin in the dark, or the apoprotein in the light. With the latter, in the light, but not in the dark, the reduction of the cytochrome decreases progressively after initial reduction, suggesting that the carotenoid may protect the cytochrome against light-sensitised oxidation.

12. The reduction of the cytochrome by ovorubin or its apoprotein is competitively inhibited by superoxide dismutase, indicating the generation of O_2^- by both ovorubin and its apoprotein in the light and the dark. Under anaerobic conditions in the dark, the reduction of cytochrome *c* by ovorubin and its apoprotein reaches about one third of the level obtained aerobically with ovorubin in the light and about half of that obtained aerobically with ovorubin or the apoprotein in the dark.

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